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(54) Title: GENETIC SEQUENCES ENCODING FLAVONOID PATHWAY ENZYMES AND USES THEREFOR

(57) Abstract

The present invention relates to a nucleic acid isolate comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, a flavonoid 3′-hydroxylase or a functional derivative thereof. The present invention also relates to transgenic plants carrying and/or expressing the above-mentioned nucleic acid material.

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GENETIC SEQUENCES ENCODING FLAVONOID PATHWAY ENZYMES AND USES THEREFOR

The present invention relates generally to genetic sequences encoding flavonoid pathway metabolising enzymes and more particularly to flavonoid 3'-hydroxylase or fragments or derivatives thereof and their use in the manipulation of pigmentation in plants and other organisms.

The flower industry strives to develop new and different varieties of flowering plants. An effective way to create such novel varieties is through the manipulation of flower colour. Classical breeding techniques have been used with some success to produce a wide range of colours for most of the commercial varieties of flowers. This approach has been limited, however, by the constraints of a particular species' gene pool and for this reason it is rare for a single species to have a full spectrum of coloured varieties. In addition, traditional breeding techniques lack precision. The aesthetic appeal of the flower is a combination of many factors such as form, scent and colour; modification of one character through hybridization can often be at the expense of an equally valuable feature. The ability to engineer precise colour changes in cutflower and ornamental species would offer significant commercial opportunities in an industry which has rapid product turnover and where novelty is an important market characteristic.

Flower colour is predominantly due to two types of pigment: flavonoids and carotenoids. Flavonoids contribute to a range of colours from yellow to red to blue. Carotenoids impart an orange or yellow tinge and are commonly the major pigment in yellow or orange flowers. The flavonoid molecules which make the major contribution to flower colour are the anthocyanins which are glycosylated derivatives of cyanidin, delphinidin, petunidin, peonidin, malvidin and pelargonidin, and are localised in the vacuole. The different anthocyanins can produce marked differences in colour. Flower colour is also influenced by co-pigmentation with colourless flavonoids, metal complexation, glycosylation, acylation and vacuolar pH (Forkmann,

1991).

The biosynthetic pathway for the flavonoid pigments (hereinafter referred to as the "flavonoid pathway") is well established and is shown in Figure 1 (Ebel and Hahlbrock, 1988; Hahlbrock and Grisebach, 1979; Wiering and De Vlaming, 1984; Schram *et al*, 1984; Stafford, 1990). The first committed step in the pathway involves the condensation of three molecules of malonyl-CoA with one molecule of *p*-coumaroyl-CoA. This reaction is catalysed by the enzyme chalcone synthase (CHS). The product of this reaction, 2',4,4',6', tetrahydroxy-chalcone, is normally rapidly isomerized to produce naringenin by the enzyme chalcone flavanone isomerase (CHI). Naringenin is subsequently hydroxylated at the 3 position of the central ring by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK).

The pattern of hydroxylation of the B-ring of DHK plays a key role in determining petal colour. The B-ring can be hydroxylated at either the 3', or both the 3' and 5' positions, to produce dihydroquercetin (DHQ) and dihydromyricetin (DHM), respectively. Two key enzymes involved in this pathway are flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase, both of the cytochrome P450 class. Cytochrome P450 enzymes are widespread in nature and genes have been isolated and sequenced from vertebrates, insects, yeasts, fungi, bacteria and plants.

Flavonoid 3'-hydroxylase acts on DHK to produce DHQ and on naringenin to produce eriodictyol. Reduction and glycosylation of DHQ produces the cyanidin-glycoside and peonidin-glycoside pigments which, in many plant species (for example rose, carnation and chrysanthemum), contribute to red and pink flower colour. The synthesis of these anthocyanins can also result in other flower colours. For example, blue cornflowers contain cyanin. The ability to control flavonoid 3'-hydroxylase activity, or other enzymes involved in the flavonoid pathway, in flowering plants would provide a means to manipulate petal colour. Different coloured versions of a single cultivar could thereby be generated and in some instances a single species would be able to produce a broader spectrum of colours.

In accordance with the present invention, the genetic sequences encoding flavonoid 3'-hydroxylase have been identified and cloned. These recombinant sequences permit the modulation of hydroxylation of substrates such as DHK and naringenin, leading to a modification of anthocyanin composition, thereby providing a means to manipulate petal colour. The presence of the flavonoid 3'-hydroxylase would allow the diversion of the metabolic pathway from DHK to anthocyanin derivatives of anthocyanidins such as cyanidin and peonidin, thereby providing a means to manipulate petal colour by modulation of the level of 3'-hydroxylation. Accordingly, the present invention relates to the altering of flavonoid 3'-hydroxylase activity in plants, which encompasses elevating or reducing levels of existing flavonoid 3'-hydroxylase activity by introducing the sequences of the present invention. Reduction in levels of flavonoid 3'-hydroxylase activity may also be referred to as down-regulation. Moreover, the present invention extends beyond flowers to fruit and vegetable plants and to leaves of, for example, ornamental plants.

Accordingly, one aspect of the present invention provides a nucleic acid isolate comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, flavonoid 3'-hydroxylase enzyme (hereinafter referred to as 3'-hydroxylase) or a functional derivative of the enzyme.

By the term "nucleic acid isolate" is meant a genetic sequence in a non-naturally-occurring condition. Generally, this means isolated away from its natural state or synthesized or derived in a non-naturally-occurring environment. More specifically, it includes nucleic acid molecules formed or maintained *in vitro*, including genomic DNA fragments, recombinant or synthetic molecules and nucleic acids in combination with heterologous nucleic acids. It also extends to the genomic DNA or cDNA or part thereof encoding 3'-hydroxylase or part thereof in reverse orientation relative to its or another promoter. It further extends to naturally-occurring sequences following at least a partial purification relative to other nucleic acid sequences.

The term "genetic sequences" is used herein in its most general sense and encompasses any contiguous series of nucleotide bases specifying directly, or via a complementary series of bases, a sequence of amino acids in a 3'-hydroxylase. Such a sequence of amino acids may constitute a full-length 3'-hydroxylase or an active truncated form thereof or may correspond to a particular region such as an N-terminal, C-terminal or internal portion of the enzyme. The nucleic acid sequences contemplated herein also encompass oligonucleotides useful as genetic probes or as "antisense" molecules capable of regulating expression of the corresponding gene in a plant. An "antisense molecule" as used herein may also encompass a gene construct comprising the structural genomic or cDNA gene or part thereof in reverse orientation relative to its or another promoter.

In one embodiment the nucleic acid sequence encoding 3'-hydroxylase or various functional derivatives thereof are used to reduce the activity of an endogenous 3'-hydroxylase, or alternatively the nucleic acid sequence encoding this enzyme or various derivatives or parts thereof are used in the antisense orientation to reduce activity of the 3'-hydroxylase. Although not wishing to limit the present invention to any one theory, it is possible that an antisense 3'-hydroxylase transcript or fragment or part thereof (for example, an oligonucleotide molecule) would form a duplex with all or part of the naturally occurring mRNA specified for the enzyme thus preventing accumulation of or translation from the mRNA into active enzyme. In a further alternative, ribozymes could be used to inactivate target nucleic acid sequences.

Reference herein to the altering of flavonoid 3'-hydroxylase activity relates to an elevation or reduction in activity of up to 30% or more preferably of 30-50%, or even more preferably 50-75% or still more preferably 75% or greater above or below the normal endogenous or existing levels of activity. The level of activity can be readily assayed using a modified version of the method described by Stotz and Forkmann (1982) (see Example 1).

The nucleic acids of the present invention may be a ribonucleic acid or deoxyribonucleic acids, single or double stranded and linear or covalently closed circular molecules. Preferably, the nucleic acid molecule is cDNA. The present invention also extends to other nucleic acid molecules which hybridize under low, preferably under medium and most preferably under high stringency conditions with the nucleic acid molecules of the present invention and in particular to the sequence of nucleotides set forth in Figure 5 or a part or region thereof. In its most preferred embodiment, the present invention extends to a nucleic acid molecule having a nucleotide sequence set forth in Figure 5 or to a molecule having at least 40%, more preferably at least 45%, even more preferably at least 55%, still more preferably at least 65-70%, and yet even more preferably greater than 85% similarity at the level of nucleotide or amino acid sequence to at least one or more regions of the sequence set forth in Figure 5 and wherein the nucleic acid encodes or is complementary to a sequence which encodes an enzyme having 3'-hydroxylase activity. It should be noted, however, that nucleotide or amino acid sequences may have similarities below the above given percentages and yet still encode 3'-hydroxylase activity and such molecules may still be considered in the scope of the present invention where they have regions of sequence conservation. The present invention further extends to nucleic acid molecules in the form of oligonucleotide primers or probes capable of hybridizing to a portion of the nucleic acid molecules contemplated above, and in particular those set forth in Figure 5, under low, preferably under medium and most preferably under high stringency conditions. Preferably the portion corresponds to the 5' or the 3' end of the gene. For convenience the 5' end is considered herein to define a region substantially between the start codon of the structural genetic sequence to a centre portion of the gene, and the 3' end is considered herein to define a region substantially between the centre portion of the gene and the terminating codon of the structural genetic sequence. It is clear, therefore, that oligonucleotides or probes may hybridize to the 5' end or the 3' end or to a region common to both the 5' and the 3' ends. The present invention extends to all such probes. Preferred oligonucleotides are set forth in Example 1.

The nucleic acid or its complementary form may encode the full-length enzyme or a part or derivative thereof. By "derivative" is meant any single or multiple amino acid substitutions, deletions, and/or additions relative to the naturally-occurring enzyme and which retains 3'-hydroxylase activity. In this regard, the nucleic acid includes the naturally-occurring nucleotide sequence encoding 3'-hydroxylase or may contain single or multiple nucleotide substitutions, deletions and/or additions to said naturally-occurring sequence. The nucleic acid of the present invention or its complementary form may also encode a "part" of the 3'-hydroxylase, whether active or inactive, and such a nucleic acid molecule may be useful as an oligonucleotide probe, primer for polymerase chain reactions or in various mutagenic techniques, or for the generation of antisense molecules.

Amino acid insertional derivatives of the 3'-hydroxylase of the present invention include amino and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Typical substitutions are those made in accordance with Table 1 overleaf.

Where the 3'-hydroxylase is derivatised by amino acid substitution, the amino acids are generally replaced by other amino acids having like properties, such as hydrophobicity, hydrophilicity, electronegativity, bulky side chains and the like. Amino acid substitutions are typically of single residues. Amino acid insertions will usually be in the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, deletions or insertions are made in adjacent pairs, i.e. a deletion of two residues or insertion of two residues.

The amino acid variants referred to above may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis (Merrifield, 1964) and the like, or by recombinant DNA manipulations. Techniques for making substitution mutations at predetermined sites in DNA having known or partially known sequence are well known and include, for example, M13 mutagenesis. The manipulation of DNA sequence to produce variant proteins which manifest as substitutional, insertional or deletional variants are conveniently described, for example, in Sambrook *et al.*(1989).

TABLE 1

Suitable residues for amino acid substitutions

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Other examples of recombinant or synthetic mutants and derivatives of the 3'-hydroxylase of the present invention include single or multiple substitutions, deletions and/or additions of any molecule associated with the enzyme such as carbohydrates, lipids and/or proteins or polypeptides.

The terms "analogues" and "derivatives" also extend to any functional chemical equivalent of the 3'-hydroxylase and also to any amino acid derivative described above. For convenience, reference to "3'-hydroxylase" herein includes reference to any mutants, derivatives, analogues, homologues or fragments thereof.

The present invention is exemplified using nucleic acid sequences derived from petunia since this represents the most convenient and preferred source of material to date. However, one skilled in the art will immediately appreciate that similar sequences can be isolated from any number of sources such as other plants or certain microorganisms. Examples of other plants include, but are not limited to, carnation, chrysanthemum, rose, maize, snapdragon, tobacco, cornflower, pelargonium and morning glory. All such nucleic acid sequences encoding directly or indirectly a flavonoid pathway enzyme and in particular 3'-hydroxylase, regardless of their source, are encompassed by the present invention.

The nucleic acid molecules contemplated herein may exist in either orientation alone or in combination with a vector molecule, for example an expression-vector. The term vector molecule is used in its broadest sense to include any intermediate vehicle for the nucleic acid molecule, capable of facilitating transfer of the nucleic acid into the plant cell and/or facilitating integration into the plant genome. An intermediate vehicle may, for example, be adapted for use in electroporation, microprojectile bombardment, *Agrobacterium*-mediated transfer or insertion via DNA or RNA viruses. The intermediate vehicle and/or the nucleic acid molecule contained therein may or may not need to be stably integrated into the plant genome. Such vector molecules may also replicate and/or express in prokaryotic cells. Preferably, the vector molecules or parts thereof are capable of integration into the plant

genome. The nucleic acid molecule may additionally contain a promoter sequence capable of directing expression of the nucleic acid molecule in a plant cell. The nucleic acid molecule and promoter may also be introduced into the cell by any number of means such as those described above.

In accordance with the present invention, a nucleic acid sequence encoding 3'-hydroxylase or a derivative or part thereof may be introduced into and expressed in a plant in either orientation thereby providing a means either to convert DHK and/or other suitable substrates, if synthesised in the plant cell, ultimately into anthocyanin derivatives of anthocyanidins such as cyanidin and/or peonidin, or alternatively to inhibit such conversion of metabolites by reducing or eliminating endogenous or existing 3'-hydroxylase activity. The production of anthocyanins contributes to the production of a red or blue flower colour. Expression of the nucleic acid sequence in either orientation in the plant may be constitutive, inducible or developmental, and may also be tissue-specific. The word expression is used in its broadest sense to include production of RNA or of both RNA and protein. It also extends to partial expression of a nucleic acid molecule.

According to this aspect of the present invention there is provided a method for producing a transgenic plant capable of synthesizing 3'-hydroxylase or active mutants or derivatives thereof, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding said 3'-hydroxylase, under conditions permitting the eventual expression of said nucleic acid molecule, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid. The transgenic plant may thereby produce elevated levels of 3'-hydroxylase activity relative to the amount expressed in a comparable non-transgenic plant.

Another aspect of the present invention contemplates a method for producing a transgenic plant with reduced endogenous or existing 3'-hydroxylase activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding 3'-hydroxylase, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the expression of the nucleic acid.

Yet another aspect of the present invention contemplates a method for producing a genetically modified plant with reduced endogenous or existing 3'-hydroxylase activity, said method comprising altering the 3'-hydroxylase gene through modification of the endogenous sequences via homologous recombination from an appropriately altered 3'-hydroxylase gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

In a preferred embodiment, the present invention contemplates a method for producing a transgenic flowering plant exhibiting altered inflorescence properties, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence into the 3'-hydroxylase enzyme. Alternatively, said method may comprise stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention or its complementary sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to alter the level of activity of the endogenous or existing 3'-hydroxylase. Preferably the altered level would be less than the endogenous or existing level of 3'-hydroxylase activity in a comparable non-transgenic plant. Without wishing to limit the present invention, one theory of mode of action is that reduction of the endogenous 3'-hydroxylase activity requires the expression of the introduced nucleic acid sequence or its complementary sequence. However, expression of the introduced genetic sequence or its complement may not be

required to achieve the desired effect: namely, a flowering plant exhibiting altered inflorescence properties.

In a related embodiment, the present invention contemplates a method for producing a flowering plant exhibiting altered inflorescence properties, said method comprising alteration of the 3'-hydroxylase gene through modification of the endogenous sequences via homologous recombination from an appropriately altered 3'-hydroxylase gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

The nucleic acid molecule of the present invention may or may not be developmentally regulated. Preferably, the altered inflorescence includes the production of red flowers or other colour shades depending on the physiological conditions of the recipient plant. By "recipient plant" is meant a plant capable of producing a substrate for the 3'-hydroxylase enzyme, or producing the 3'-hydroxylase enzyme itself, and possessing the appropriate physiological properties and genotype required for the development of the colour desired. This may include but is not limited to petunia, carnation, chrysanthemum, rose, snapdragon, tobacco, cornflower, pelargonium, lisianthus and morning glory.

Accordingly, the present invention extends to a method for producing a transgenic plant capable of expressing a recombinant gene encoding 3'-hydroxylase or part thereof or which carries a nucleic acid sequence which is substantially complementary to all or a part of a mRNA molecule optionally transcribable where required to effect regulation of a 3'-hydroxylase, said method comprising stably transforming a cell of a suitable plant with the nucleic acid isolate comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, 3'-hydroxylase or a derivative or part thereof, where necessary under conditions permitting the eventual expression of said nucleic acid isolate, and regenerating a transgenic plant from the cell.

One skilled in the art will immediately recognise the variations applicable to the methods of the present invention, such as increasing or decreasing the expression of the enzyme naturally present in a target plant leading to differing shades of colours such as different shades of red.

The present invention, therefore, extends to all transgenic plants containing all or part of the nucleic acid sequence of the present invention and/or any homologues or related forms thereof or antisense forms of any of these and in particular those transgenic plants which exhibit altered inflorescence properties. The transgenic plants may contain an introduced nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding 3'-hydroxylase. Generally the nucleic acid would be stably introduced into the plant genome, although the present invention also extends to the introduction of the 3'-hydroxylase nucleotide sequence within an autonomously-replicating nucleic acid sequence such as a DNA or RNA virus capable of replicating within the plant cell. The invention also extends to seeds from such transgenic plants. Such seeds, especially if coloured, will be useful as proprietary tags for plants.

A further aspect of the present invention is directed to recombinant forms of 3'-hydroxylase. The recombinant forms of the enzymes will provide a source of material for research to develop, for example, more active enzymes and may be useful in developing *in vitro* systems for production of coloured compounds.

Still a further aspect of the present invention contemplates the use of the genetic sequences described herein in the manufacture of a genetic construct capable of expressing a 3'-hydroxylase enzyme or down-regulating an endogenous 3'-hydroxylase in a plant.

The present invention is further described by reference to the following non-limiting Figures and Example.

In the Figures:

Figures 1 is a schematic representation of the biosynthesis pathway for the flavonoid pigments. Enzymes involved in the first part of the pathway have been indicated as follows: PAL = Phenylalanine ammonia-lyase; C4H = Cinnamate 4-hydroxylase; 4CL = 4-coumarate: CoA ligase; CHS = Chalcone synthase; CHI = Chalcone flavanone isomerase; F3H = Flavanone 3-hydroxylase; DFR = Dihydroflavonol-4-reductase; UFGT = UDP-glucose: flavonoid-3-O-glucosyl-transferase. The later steps correspond to conversions that occur in *P. hybrida* flowers and include: 1 = addition of a rhamnose sugar to the glucosyl residue of cyanidin-3-glucoside and delphinidin-3-glucoside; 2 = acylation and 5-O-glucosylation; 3 = 3' methylation; 4 = 5' methylation; 5 = 3',5' methylation.

Figure 2(A) is a schematic representation of DNA fragments used to probe cDNA library #1 to identify cytochrome P450 homologues. **P450**: generalized cytochrome P450 cDNA clone with the haem-binding domain (Haem) indicated by the shaded box; **pCGP142**: a 980 bp fragment was obtained by PCR with oligos 1 and 2 using pCGP142 DNA as template; **pCGP147**: a 1.3 kb fragment was isolated from a SalI-EcoRI digest of pCGP147; **pCGP158**: a 900 bp fragment was obtained by PCR with oligos 3 and 4 using pCGP158 DNA as template; **pCGP160**: a 600 bp fragment was isolated from a PstI-EcoRV digest of pCGP160; **pCGP454**: fragment was obtained by PCR with oligos 3 and 5 using pCGP454 DNA as template. All purified fragments were labelled with ^{32}P-dCTP as described in Example 1.

Figure 2(B) to **(H)** show partial nucleotide sequences and the corresponding predicted amino acid translation products for the cDNA inserts from (i) pCGP142, (ii) pCGP147, (iii) pCGP158, (iv) pCGP160 and (v) pCGP454. The regions used to probe cDNA library #1 to isolate related clones have been delineated by arrowheads.

Figure 3(A) to (D) is the nucleotide sequence and predicted amino acid sequence for the cDNA insert from pCGP602. Two probes that included the sequences between the internal HincII-EcoRV and EcoRV-HindIII sites were used to identify related sequences in a group of cytochrome P450 homologues.

Figures 4(A) and 4(B) show partial nucleotide sequence for the cDNA inserts from: 4(A): 1) pCGP161; 2) pCGP162; 3) pCGP163; 4) pCGP165; 5) pCGP166; 6) pCGP167, and 4(B): 7 pCGP168; 8) pCGP169; 9) pCGP171 and 10) pCGP173. A mixed probe that included the cDNA inserts of all these clones was used to screen cDNA library #2 for related sequences.

Figure 5 is the nucleotide sequence and predicted amino acid sequence for the cDNA insert from pCGP619.

Figure 6 shows a diagrammatic representation of a restriction enzyme map of pCGP619. Partial lengths of the cDNA insert are indicated by the bolder lines with solid ends (as opposed to arrows). These were subcloned into M13-mp18 and mp19 and sequenced using oligonucleotide primer sequences, as indicated, to obtain overlapping sequence information. The extent and direction of sequence information obtained from each subcloned piece is shown by lines with arrowheads. Primer -40 was used unless otherwise specified. 190 = primer sequence 190; 191 = primer sequence 191; poly T = poly T oligonucleotide was used as primer; ds seq = sequence was read with double-stranded DNA; ATG indicates the methionine initiation codon and the total length of the clone in base pairs is also indicated.

Figure 7 shows a 3'-hydroxylase assay of yeast extracts using ^{3}H-naringenin as substrate. The autoradiograph shows conversion of ^{3}H-naringenin to the 3'-hydroxylated derivative eriodictyol by an extract of yeast transformed with the plasmid pCGP621 (1, 2). No 3'-hydroxylase activity was detected in untransformed yeast (C).

Figure 8 shows nucleotide sequence and predicted amino acid sequence for the insert from pCGP635. These sequences may be used as probes for the isolation of putative rose 3'-hydroxylase cDNA clones.

Figure 9 shows nucleotide sequence and predicted amino acid sequence for the insert from pCGP772. These sequences may be used as probes for the isolation of putative carnation 3'-hydroxylase cDNA clones.

Figure 10 shows nucleotide sequence and predicted amino acid sequence for the insert from pCGP773. These sequences may be used as probes for the isolation of carnation putative 3'-hydroxylase cDNA clones.

Figure 11 shows partial nucleotide sequence and predicted amino acid sequence for insert from pCGP854. These sequences were used as a probe to select a putative 3'-hydroxylase cDNA clone. Underlined amino acids are identical to those of the cDNA insert from pCGP619 between positions 971 and 1091.

The disarmed microorganism *Agrobacterium tumefaciens* strain AGL0 containing the plasmid pCGP809 was deposited with the Australian Government Analytical Laboratories, 1 Suakin Street, Pymble, New South Wales, 2037, Australia on March 24, 1993 and was given Accession Number

EXAMPLE 1

ISOLATION OF 3'-HYDROXYLASE AND RELATED NUCLEIC ACID SEQUENCES

1. Materials and methods

Chemicals Enzymes and Radioisotopes

Eriodictyol was obtained from Carl Roth KG and naringenin was obtained from Sigma. [^{3}H]-Naringenin (5.7 Ci/mmole) was obtained from Amersham. All enzymes were obtained from commercial sources and used according to the manufacturer's recommendations.

Bacterial Strains

The *Escherichia coli* strains used were:

DH5a — supE44, Δ(lacZYA-ArgF)U169, ϕ80lacZΔM15, hsdR17 (r_k^-, m_k^+), recA1, endA1, gyrA96, thi-1, relA1, deoR. (Hanahan, 1983 and BRL, 1986).

XL1-Blue — supE44, hsdR17 (r_k^-, m_k^+), recA1, endA1, gyrA96, thi-1, relA1, lac-, [F'proAB, lacI^q, lacZΔM15, Tn10(tet^r)] (Bullock *et al*,1987).

PLK-F' — recA, hsdR17 (r_k^-, m_k^+), mcrA$^-$, mcrB$^-$, lac$^-$, supE44, galK2, galT22, metBl, [F' proAB, lacI^q, lacZΔM15, Tn10(tet^r)] (Stratagene).

SOLR — e14$^-$ (mcrA), Δ(mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, umuC::Tn5(kan^r), uvrC,lac, gyrA96, thi-1, relA1, [F'proAB, lacI^{q}ZΔM15], Su$^-$ (non-suppressing) (Stratagene)

The disarmed *Agrobacterium tumefaciens* strain AGL0 (Lazo et al., 1991) was obtained from R Ludwig (Department of Biology, University of California, Santa Cruz).

The cloning vector pBluescript was obtained from Stratagene.

Transformation of *E. coli* and *A. tumefaciens*

Transformation of the E. coli strain DH5a cells was performed according to the method of Inoue *et al.* (1990).

The plasmid pCGP809 was introduced into the *Agrobacterium tumefaciens* strain AGL0 by adding 5 mg of plasmid DNA to 100 mL of competent AGL0 cells prepared by inoculating a 50 mL MG/L (Garfinkel and Nester, 1980) culture and growing for 16 h with shaking at 28°C. The cells were then pelleted and resuspended in 0.5 mL of 85% (v/v) 100 mM $CaCl_2$/15% (v/v) glycerol. The DNA-*Agrobacterium* mixture was frozen by incubation in liquid N_2 for 2 min and then allowed to thaw by incubation at 37°C for 5 min. The DNA/bacterial mixture was then placed on ice for a further 10 min. The cells were then mixed with 1 mL of MG/L media and incubated with shaking for 16 h at 28°C. Cells of *A. tumefaciens* carrying pCGP809 were selected on MG/L agar plates containing 100 mg/mL gentamycin. The presence of pCGP809 was confirmed by Southern analysis of DNA isolated from the gentamycin-resistant transformants.

Plant Material

Seed of the *Petunia* F_1 hybrid "Old Glory Blue" (OGB) was obtained from Ball Seed, USA.

Chrysanthemum morifolium cultivars were obtained from Baguley Flower and Plant Growers, Victoria.

Flowers of *Dianthus caryophyllus* cv. Laguna and *Rosa hybrida* cv. Kardinal were obtained from Van Wyk and Son Flower Supply, Victoria.

Plants were grown in specialised growth rooms with a 14 hr day length at a light intensity of 10,000 lux minimum and a temperature of 22 to 26°C.

Five stages of *Petunia* flower development were defined as follows:

Stage 1: Unpigmented, closed bud (<25 mm in length).
Stage 2: Pigmented, closed bud (25-35 mm in length).
Stage 3: Dark purple bud with emerging corolla (>35 mm in length).
Stage 4: Dark purple opened flower pre-anther dehiscence (>50 mm in length).
Stage 5: Fully opened flower with all anthers dehisced.

Stages of *Chrysanthemum* flower development were defined as follows:

Stage 0: No visible flower bud.
Stage 1: Flower bud visible: florets completely covered by the bracts.
Stage 2: Flower buds opening: tips of florets visible.
Stage 3: Florets tightly overlapped.
Stage 4: Tips of nearly all florets exposed; outer florets opening but none horizontal.
Stage 5: Outer florets horizontal.
Stage 6: Flower approaching maturity.

Stages of *Dianthus caryophyllus* flower development were defined as follows:

Stage 1: No visible flower bud.
Stage 2: Flower buds opening: tips of florets visible.
Stage 3: Tips of nearly all florets exposed; outer florets opening, none horizontal.

Stage 4: Outer florets horizontal.

Stages of *Rosa hybrida* flower development were defined as follows:

Stage 1: Unpigmented, tightly closed bud (10-12mm high; 5mm wide).

Stage 2: Pigmented, tightly closed bud (15mm high ; 9mm wide).

Stage 3: Pigmented, closed bud; sepals just beginning to open (20-25mm high; 13-15mm wide)

Stage 4: Flower bud beginning to open; petals heavily pigmented; sepals have separated (bud is 25-30mm high and 18mm wide).

Stage 5: Sepals completely unfolded; some curling. Petals are heavily pigmented and unfolding (bud is 30-33mm high and 20mm wide).

Construction of cDNA library #1

Twenty grams of stage 3 to 4 *Petunia* cv. OGB flower limbs tissue was homogenised in 100 mL of PEB (200 mM Tris-HCl (pH 8.6), 60 mM KCl, 30 mM $MgCl_2$, 25 mM EGTA) containing 10 mM vanadyl ribonucleoside complex. Cell debris was removed by filtering the homogenate through sterile Miracloth (Calbiochem). The filtrate was layered on top of a step gradient of 6 mL of PEB containing 25% (w/v) sucrose, 250 units InhibitAce (5-Prime 3-Prime), and 6 mL of PEB containing 50% (w/v) sucrose and 250 units InhibitAce in Ultra-Clear™ Quick-Seal™ (Beckman) centrifuge tubes. The tubes were centrifuged for 3.5 h at 26,000 rpm in a 70Ti rotor. Membrane-bound polysomes were collected from the 25% sucrose/50% sucrose interface and added to a 4 M guanidium isothiocyanate solution. RNA was isolated from the denatured polysomes by pelleting through a 5.7 M CsCl cushion, as described by Turpen and Griffith (1986).

A Uni-ZAP™ XR vector kit (Stratagene) was used to construct a directional cDNA library in lZAP using 25 µg of the polysomal RNA as template. The primary library, which contained 250,000 plaque forming units (pfu), was amplified by overnight growth on NZY plates (Sambrook *et al.*, 1989) and the amplified phage stock was eluted in PSB (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl (pH 7.5), 0.01%

(w/v) gelatin) as described by Sambrook *et al*, (1989).

Construction of cDNA library #2

Total RNA was isolated from the petal tissue of *P. hybrida* cv. OGB stage 3 to 4 flowers using the method of Turpen and Griffith (1986). Poly(A)$^+$ RNA was selected from the total RNA by three cycles of oligo-dT cellulose chromatography (Aviv and Leder, 1972).

Two micrograms of poly(A)$^+$ RNA were reverse transcribed in a 20 µL volume containing 1 x Superscript™ reaction buffer, 10 mM dithiothreitol (DTT), 500 µM dATP, 500 µM dGTP, 500 µM dTTP, 500 µM 5-methyl-dCTP, 0.75 µg oligonucleotide #6 and 2 µL Superscript™ reverse transcriptase (BRL). The reaction mix was incubated at 37°C for 50 min, 44°C for 10 min, then placed on ice.

Second strand reaction mix (140 µL) was added to the first strand reaction. The second strand reaction mix consisted of 21 mM Tris-HCl, 104 mM KCl, 5.3 mM $MgCl_2$, 171 µM b-NAD, 11.4 mM $(NH_4)_2SO_4$, 214 µM dATP, 642 µM dCTP, 214 µM dGTP, 214 µM dTTP, 4 mM DTT, 10 µCi ^{32}P-dCTP (3000 Ci/mMole), 15 units *E.coli* DNA ligase, 40 units DNA polymerase (Boehringer) and 0.8 units RNAse H. The final mixture was incubated for 150 min at 16°C. To make the double-stranded cDNA blunt-ended, 10 units T4 DNA polymerase was added, and the reaction continued for a further 15 min at 16°C. The reaction was stopped and the cDNA purified by phenol/chloroform extraction, followed by chloroform extraction and ethanol precipitation.

EcoRI adaptors (Promega) were ligated with the cDNA and then kinased using conditions recommended by the manufacturer. The enzymes were denatured by heat (70°C, 20 min) and the DNA was purified by phenol/chloroform extraction and ethanol precipitation. The cDNA was digested with 50 units XhoI (Boehringer) in a reaction volume of 100 µL, using conditions recommended by the manufacturer. The enzyme was heat killed (70°C, 20 min) and the mixture passed through an S400 spun column (Pharmacia) which had been equilibrated in STE buffer (Sambrook *et*

al, 1989).

The eluate was phenol/chloroform extracted and ethanol precipitated. After microcentrifugation at 4°C for 30 min the cDNA pellet was rinsed with 70% (v/v) ethanol, air dried and resuspended in 10 µL of TE buffer (10 mM Tris-HCl (pH7.5), 1 mM EDTA).

NA-45 membrane (Schleicher and Schuell) was used to isolate cDNA in the size range of 1.3 to 2.5kb from a 7.5 µL sample that had been electrophoresed through a 1% (w/v) agarose gel.

The size fractionated cDNA was ligated with 1 µg λZAPII EcoRI/XhoI/ CIAP treated vector (Stratagene) in 5 µL reaction buffer consisting of 50 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 2 units T4 DNA ligase. The reaction was performed at 4°C for 2 days.

After leaving at room temperature for 2 h, the ligation reaction mixture was packaged using the Packagene system (Promega). The total number of recombinants was 270,000 pfu.

An amount of 150,000 pfu of the packaged cDNA was plated at 10,000 pfu per 15 cm diameter plate after transfecting PLK-F' cells. The plates were incubated at 37°C for 8 h, then stored overnight at 4°C. Duplicate lifts were taken onto Colony/Plaque Screen™ filters (DuPont) and treated as recommended by the manufacturer.

Construction of cDNA library #3

Total RNA was isolated from the petal tissue of *Chrysanthemum morifolium* cv. Dark Pink Pompom (Reference Number 5999), stages 1, 2 and 3 flowers, again using the method of Turpen and Griffith (1986). Poly(A)$^+$ RNA was selected from the total RNA, as for *P. hybrida*, by three cycles of oligo-dT cellulose chromatography (Aviv and Leder, 1972). Two micrograms of poly(A)$^+$ RNA were used as template for cDNA synthesis, as outlined above for *P. hybrida*.

Following fractionation and ligation, the cDNA reaction mixture was packaged using the Packagene system (Promega). The total number of recombinants was 37,000 pfu.

An amount of 300,000 pfu (of amplified library) of the packaged cDNA was plated at 20,000 pfu per 15 cm diameter plate after transfecting XL1-Blue cells. The plates were incubated at 37°C for 8 h, then stored overnight at 4°C. Duplicate lifts were taken onto Colony/Plaque Screen™ filters (DuPont) and treated as recommended by the manufacturer.

Preparation of PCR templates

1. Plasmid DNA

DNA was isolated using an alkaline lysis procedure (Sambrook *et al*; 1989). Plasmid DNA was further purified by banding on a CsCl gradient. This DNA was used as template for PCR.

2. *Chrysanthemum* genomic DNA

For isolation of total DNA, 5 g of *Chrysanthemum* petal tissue was frozen in liquid nitrogen and ground to a fine powder in a cold mortar and pestle. Ground tissue was extracted in 5 mL of phenol:chloroform, followed by 5 mL of NTMES buffer (0.01 M NaCl; 0.1 M Tris pH 8.5; 5 mM $MgCl_2$; 1 mM EDTA; 1% SDS). The aqueous phase was re-extracted with 5 mL of phenol:chloroform and the aqueous phase collected after centrifugation. DNA was spooled from this solution after addition of 0.5 mL 3M NaAc, pH 5.8 and two volumes of ethanol. The final pellet was resuspended in 2 mL TE buffer and the concentration determined prior to use in PCR.

3. *Dianthus* cDNA

Total RNA was isolated from the petal tissue of *D. caryophyllus* cv. Laguna stage 3 flowers, likewise using the method of Turpen and Griffith (1986). Poly(A)$^+$ RNA was selected from the total RNA by Oligotex dT-30 (Takana, Japan) following the manufacturer's protocol, and two micrograms were reverse transcribed using

Superscript™ reverse transcriptase as recommended by the manufacturer. The cDNA was dissolved in 10 mL TE buffer. For PCR reactions, 5 mL were used as template. Conditions for PCR are described below.

4. *Rosa* cDNA

Total RNA was prepared from the buds of *Rosa hybrida* cv. Kardinal stage 1. At this stage, buds were 1.0 - 1.2 cm high and approximately 0.5 cm wide. They were completely closed and no pigment was visible when the sepals were dissected away.

Frozen tissue (1-3 g) was ground in liquid nitrogen with a mortar and pestle, placed in 25 mL pre-chilled Buffer A [0.2 M boric acid, 10 mM EDTA (sodium salt) (pH 7.6)] and homogenized briefly. The extract was mixed on a rotary shaker until it reached room temperature and an equal volume of phenol/chloroform (1:1 v/v), equilibrated with Buffer A, was added. After mixing for a further 10 min, the RNA preparation was centrifuged at 10,000 x *g* for 10 min at 20° C. The upper aqueous phase was retained and the phenol interface re-extracted as above. The aqueous phases were pooled and adjusted to 0.1 M sodium acetate (pH 6.0), 2.5 volumes 95% ethanol were added and the mixture was stored at -20° C overnight.

The preparation was centrifuged at 10,000 x *g* for 10 min at 4° C, the pellet dissolved gently in 20 mL Buffer B [25 mM boric acid, 1.25 mM EDTA (sodium salt), 0.1 M NaCl (pH 7.6)] and 0.4 volumes 2-butoxyethanol (2BE) were added. This solution was incubated on ice for 30 min. It was then centrifuged at 10,000 x *g* for 10 min at 0° C and the supernatant carefully collected. After addition of 1.0 volume of 2BE and incubation on ice for a further 30 min, the supernatant was again centrifuged at 10,000 x *g* for 10 min at 0° C. The resulting pellet was gently washed with Buffer A : 2BE (1 : 1 v/v), then with 70% (v/v) ethanol, 0.1 M potassium acetate and finally with 95% ethanol. The pellet was air dried and dissolved in 1 mL diethyl pyrocarbonate (DEPC)-treated water. This was adjusted to 3 M lithium chloride, left on ice for 60 min and centrifuged at 10,000 x *g* for 10 min at 0°C. The pellet was washed twice with 3 M LiCl and then with 70% ethanol, 0.1 M potassium acetate.

The resulting RNA pellet was dissolved in 400 mL DEPC-treated water and extracted with an equal volume phenol/chloroform. The RNA mix was then centrifuged at 10,000 x *g* for 5 min at 20° C, the aqueous phase collected and made to 0.1 M sodium acetate, and a further 2.5 volumes of 95% ethanol were added. After 30 min incubation on ice, the mix was centrifuged at 13,000 rpm (5,000 x *g*) for 20 min at 20° C and the RNA pellet resuspended gently in 400 mL DEPC-treated water.

Poly $(A)^+$ RNA was selected from the total RNA by Oligotex dT-30 (Takana, Japan) following the manufacturer's protocol.

Double-stranded cDNA was synthesized from 2 mg poly$(A)^+$ RNA using the same method as described above for the construction of the *Petunia* cDNA library # 2. The cDNA was dissolved in 10 mL TE buffer.

Synthesis of Oligonucleotides

Oligonucleotides and primers were synthesized on an Applied Biosystems PCR-Mate DNA synthesizer using methods recommended by the manufacturer. The oligonucleotides and primers synthesized were, 5'-3':

Oligo 1: GTTCAATTCGGAATGATG

Oligo 2: GCTGCACTTAATCCATAT

Oligo 3: GGATGACTCAAACAGCTATGACCATG

Oligo 4: TGCATAGCTTTTGGG

Oligo 5: CCIGG(A/G)CAIATIC(G/T)(C/T)(C/T)TICCIGCICC(A/G)AAIGG

Oligo 6:

GAGAGAGAGAGAGAGAGAGATCTCGAGTTTTTTTTTTTTTTTT

Oligo 7: CCIGC(A/G)CAIATIC(G/T)IC(T/G)ICCIGCICC(A/G)AAIGG

primer -40 GTTTTCCCAGTCACGAC

primer 190 TTGGAGTGGGCAATGGC

primer 191 CTGCTGCAAACAAGTCC

poly-T TTTTTTTTTTTTTTTT(AGC)

The basis for the design of oligo 5 was as follows: Amino acid sequences from the putative haem-binding domain of an avocado cytochrome P450 (Bozak *et al.*, 1990) and the corresponding sequences encoded by the two petunia cytochrome P450 homologues pCGP142 and pCGP147 were aligned:

avocado	P	F	G	A	G	R	R	G	C	P	G
pCGP142	P	F	G	A	G	K	R	I	C	P	G
pCGP147	P	F	G	S	G	R	R	I	C	P	G

The consensus amino acid sequence of the haem-binding region for the three plant cytochromes P450 could thereby be seen to be:

P F G A(S) G R(K) R I(G) C P G

Possible permutations of nucleotide sequence that could encode the amino acids found in the haem-binding domain of the three cytochrome P450 molecules could then be deduced:

```
5'- CCX TTT GGX GCX  GGX AGX CGX ATX TGT CCX GGX -3'
          C     AG       CA  A   GG    C
                T
```

X indicates nucleotide positions where all four nucleotides (A,C,G and T) can be used. Oligo 5 was designed to complement a subset of the consensus sequence derived from the three plant cytochromes P450. Deoxyinosine (I) was used predominantly when base degeneracy was greater than three. The resulting oligonucleotide sequence was as shown above.

Polymerase Chain Reactions

1. Amplification of cloned cytochrome P450 sequences

For amplification of cloned *Petunia* cytochrome P450 sequences, PCR mixes contained 100 ng of plasmid template, 10 mM Tris- HCl (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$, 0.2 mM each dNTP, 1.0 µM each primer and 0.5 unit AmpliTaq DNA Polymerase (Cetus). Reaction mixes (100 µl) were cycled 30 times between 95°C for 1 min, 42°C for 1 min and 72°C for 2 min.

2. Amplification of *Dianthus* sequences related to *Petunia* 3'- hydroxylase

PCR mixes contained 100 ng of cDNA template, 10 mM Tris- HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM each dNTP, 1.0 µM each primer and 5 units AmpliTaq DNA Polymerase (Cetus). Reaction mixes (100 ml) were cycled firstly through 95°C for 3 min, 55°C for 1 min and 72°C for 1 min, then through a further 39 cycles between 95°C, 55°C and 72°C each for 1 min. Amplified products were gel-purified using Seaplaque low melting agarose (FMC). The mixture was heated until the agarose melted and extracted with TE-saturated phenol. The aqueous phase was then extracted with phenol/chloroform and the amplified products precipated with ethanol. Following gel-purification, the amplified products were cloned directly into the ddT-tailed pBluescript vector described by Holton and Graham (1991).

3. Amplification of *Chrysanthemum* sequences related to *Petunia* 3'-hydroxylase

Chrysanthemum reaction mixes contained 200 ng of genomic DNA template, 10 mM Tris- HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM each dNTP, 1.0 µM each primer and 5 units AmpliTaq DNA Polymerase (Cetus). Reaction volumes of 50 mL were cycled 35 times between 95°C, 55°C and 72°C, each for 90 s. Amplified products were gel-purified using Geneclean (Bio 101 Inc.) and cloned directly into the ddT-tailed pBluescript vector described by Holton and Graham (1991).

4. Amplification of *Rosa* sequences related to *Petunia* 3'-hydroxylase

Rosa reaction mixes contained 1 µL of a 10-fold dilution of ds cDNA prepared as described above, 10 mM Tris- HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM each dNTP, 1.0 µM each primer and 5 units AmpliTaq DNA Polymerase (Cetus). Reaction volumes of 50 mL were cycled 30 times between 95°C for 1 min, 55°C for 1 min and 72°C for 3 min. Amplified products were gel-purified using Geneclean (Bio 101 Inc.) and cloned directly into the ddT-tailed pBluescript vector described by Holton and Graham (1991).

Screening of cDNA Libraries

Duplicate plaque lifts from cDNA library #2 were hybridized and washed as follows: High stringency conditions (hybridization: 50% (v/v) formamide, 6 x SSC, 1% (w/v) SDS at 42°C for 16 h and washing: 2 x SSC, 1% SDS at 65°C for 2 x 15 min followed by 0.2 x SSC, 1% SDS at 65°C for 2 x 15 min) were used to detect sibling clones and low stringency conditions (hybridization: 20% formamide, 6 x SSC, 1% SDS at 42°C for 16 hand washing: 6 x SSC, 1% SDS at 65°C for 1 h) were used to detect related sequences.

Lifts from cDNA library #3 were hybridized and washed as follows: For the primary screening, using the *Petunia* 3'-hydroxylase cDNA EcoRI-XhoI insert from pCGP619 (see Figure 6), hybridization conditions were 20% (v/v) formamide, 1 M NaCl, 10% (w/v) dextransulphate at 37°C for 16 h and washing conditions were 0.1 x SSC, 1% (w/v) SDS at room temperature. For the secondary screening, using the EcoRI-XhoI insert from pCGP854, conditions were identical except that the hybridization reaction took place at 42°C for 16 h.

^{32}P-Labelling of DNA Probes

DNA fragments (50 to 100 ng) were radioactively labelled with 50 µCi of [a-^{32}P]-dCTP using an oligolabelling kit (Bresatec). Unincorporated [a-^{32}P]-dCTP was removed by chromatography on a Sephadex G-50 (Fine) column.

DNA Sequence Analysis

DNA sequencing was performed essentially by the method of Sanger *et al.* (1977) using the Sequenase enzyme (USB, version 2.1). The complete sequence of clones pCGP602 and pCGP619 was determined by compilation of sequence from different M13 -mp18 and -mp19 (Norrander *et al.*, 1983; Yanisch-Perron, 1985) subclones obtained using standard cloning procedures (Sambrook *et al.*, 1989). For some regions it was necessary to synthesise specific oligonucleotide primers to obtain overlapping sequence data, including primers -40, 190 191 and poly-T.

A restriction map of pCGP619 showing the position of several of these sequences may be seen in Figure 6.

Homology searches against Genbank, SWISS-PROT and EMBL databases were performed using the FASTA and TFASTA programs (Pearson and Lipman, 1988).

3'-Hydroxylase Assay

3'-Hydroxylase enzyme activity was measured using a modified version of the method described by Stotz and Forkmann (1982). The assay reaction mixture typically contained 100 µL of yeast extract, 5 µL of 50 mM NADPH in assay buffer (100 mM potassium phosphate (pH8.0), 1 mM EDTA and 20 mM 2-mercaptoethanol) and 10 µCi of [^{3}H]- naringenin and was made up to a final volume of 210 µL with the assay buffer. Following incubation at 23°C for 2-16 h, the reaction mixture was extracted with 0.5 mL of ethylacetate. The ethylacetate phase was dried under vacuum and then resuspended in 10 µL of ethylacetate. The tritiated flavonoid molecules were separated on cellulose thin layer plates (Merck Art 5577, Germany) using a chloroform: acetic acid: water (10:9:1 v/v) solvent system. At the completion of the chromatography the TLC plates were sprayed with 7% 2,5-diphenyloxazol in diethyl ether. The reaction products were localised by autoradiography and identified by comparison to non-radioactive naringenin and eriodictyol standards which were run alongside the reaction

products and visualised under UV light.

Construction of pCGP621

A 1.8 kb EcoRI-XhoI fragment that included the entire cDNA insert from pCGP619 was ligated with the 8 kb EcoRI-SalI fragment from pYHCC101 (Tanaka *et al*, 1988). The resulting plasmid, pCGP621, contained the pCGP619 cDNA fragment ligated in a sense orientation behind the yeast glyceraldehyde-3-phosphate dehydrogenase promoter.

Yeast transformation

The yeast strain G-1315 (Mat a, trp1) (Ashikari *et al*, 1989) was transformed with pCGP621 according to Ito *et al* (1983). The transformants were selected by their ability to restore G-1315 to tryptophan prototrophy.

Preparation of yeast extracts for assay of 3'-hydroxylase activity

A single isolate of G-1315/pCGP621 was used to inoculate 20 ml of YNBC [1.2% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose and 0.3% (w/v) casamino acid (Difco)] which was subsequently incubated for 2 days at 30°C. Cells were collected by centrifugation, washed once with TE buffer, once with buffer A [10 mM Tris-HCl (pH 7.5), 0.65 M sorbitol, 0.1 mM DTT, 0.1 mM EDTA], and then resuspended in buffer B [10 mM Tris-HCl, (pH7.5), 1.2 M sorbitol, 0.1 mM DTT, 0.1 mM EDTA] containing zymolyase (0.1 mg/mL) (Seikagakukogyo, Japan). Following incubation for 1 h at 30°C the cells were pelleted by centrifugation and resuspended in 400 µL of buffer A. The cell suspension was then vortexed with glass beads (diameter = 0.4 mm) for 2 min and a 100 µL sample was assayed for activity.

Construction of pCGP293

The expression binary vector pCGP293 was derived from the Ti binary vector pCGN1559 (McBride and Summerfelt, 1990). Plasmid pCGN1559 was digested with KpnI and the overhanging 3' ends were removed with T4 DNA polymerase according to standard protocols (Sambrook *et al*,1989). The vector was then

further digested with XbaI and the resulting 5' overhang was repaired using the Klenow fragment of DNA polymerase I. The vector was then re-ligated to give pCGP67. A 1.97 kb PstI fragment containing the Mac promoter, mas terminator and various cloning sites (Comai *et al*, 1990) was isolated from pCGP40 and inserted into the PstI site of pCGP67 to give pCGP293.

Plasmid pCGP40 was constructed by removing the GUS gene (Jefferson *et al*, 1987) as a BamHI-SacI fragment from pCGN7334 and replacing it with the BamHI-SacI fragment from pBluescribe M13⁻ that includes the multicloning site. Plasmid pCGN7334 (obtained from Calgene Inc., CA, USA), was constructed by inserting the fragment containing the Mac-GUS-mas gene fusion into the XhoI site of pCGN7329 (Comai *et al*, 1990).

Construction of pCGP809

Plasmid pCGP809 was constructed by cloning the cDNA insert from pCGP619 in a sense orientation behind the Mac promoter (Comai *et al*, 1990) of pCGP293. The 1.8 kb BamHI-KpnI fragment containing the cDNA insert was isolated from pCGP619 and ligated with a BamHI-KpnI digest of pCGP293. Correct insertion of the insert in pCGP809 was established by restriction analysis of DNA isolated from gentamycin-resistant transformants.

***Petunia* Transformation**

a. Plant material

Petunia hybrida (Skr4 x Sw63) seeds were sterilized in 1.25% (w/v) sodium hypochlorite for 10 minutes and rinsed three times in sterile water. Sterilized seeds were soaked in 100 mg/L gibberellic acid (GA_3) solution for 16 to 20 h. They were then germinated for 2 weeks on 10% (w/v) MS (Murashige and Skoog, 1962) medium supplemented with 1% (v/v) sucrose and 0.8% (w/v) Difco Bacto agar. Young seedlings were transferred to MS medium supplemented with 3% (w/v) sucrose for 3 weeks before being transferred to Jiffy peat pellets (Jiffy Products Ltd, Norway), kept under mist and illuminated (135 µE. mercury halide light, 22°C) for 2 to 3 weeks. These young plants were then transferred to a growth

cabinet (68 µE. cool white fluorescent light, 25°C). For co-cultivation, young leaves were harvested and sterilized in 1.3% (w/v) sodium hypochlorite for 2 min followed by rinsing three times in sterile water. Leaf tissue was then cut into 25 mm^2 squares and precultured on MS media supplemented with 0.05 mg/L kinetin and 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) for 24 h.

b. Co-cultivation of *Agrobacterium* and *Petunia* tissue

Agrobacterium tumefaciens strain AGL0 (Lazo *et al*, 1991) containing the binary vector pCGP809 was maintained at 4°C on MG/L (Garfinkel and Nester, 1980) agar plates with 100 mg/L gentamycin. A single colony was grown overnight in liquid medium containing 1% (w/v) Bacto-peptone, 0.5% (w/v) Bacto-yeast extract and 1% (w/v) NaCl. A final concentration of 5×10^8 cells/mL was prepared the next day by dilution in liquid MS medium containing 3% (w/v) sucrose (BPM). Leaf discs were dipped for 5 min into BPM containing AGL0/pCGP809. The leaf discs were then blotted dry and placed on co-cultivation media for 4 days. The co-cultivation medium consisted of SH medium (Schenk and Hilderbrandt, 1972) supplemented with 0.05 mg/L kinetin and 1.0 mg/L 2,4-D and included a feeder layer of tobacco cell suspension spread over the co-cultivation medium with a filter paper placed on top of the tobacco cell suspension.

c. Recovery of transgenic petunia plants

After co-cultivation, the leaf discs were transferred to selection media consisting of fresh MS medium supplemented with 3% (w/v) sucrose, 2 mg/L a-benzylaminopurine (BAP), 100 mg/L kanamycin, 350 mg/L cefotaxime, 0.3% (w/v) Gelrite Gellan Gum (Schweizerhall). After 3 weeks, regenerating explants were transferred to fresh medium. Adventitious shoots which survived the kanamycin selection were isolated and transferred to BPM containing 100 mg/L kanamycin and 350 mg/L cefotaxime for root induction. All cultures were maintained under a 16 h photoperiod (60 µE. cool white fluorescent light) at 23 ± 2°C. When roots reached 2-3 cm in length the transgenic petunia plantlets were transferred to autoclaved Debco 51410/2 potting mix in 8 cm tubes. After 4 weeks plants were replanted into 15 cm pots using the same potting mix and maintained

at 23°C under a 14 h photoperiod (300 µE. mercury halide light).

2. Results

Isolation of cytochrome P450 homologues from cDNA library #1

The isolation of five petunia cDNA clones that have regions of sequence similarity with cytochrome P450 enzymes has been described previously (International Patent Application No. PCT/AU92/00334). Partial sequences of these clones, designated pCGP142, pCGP147, pCGP158, pCGP160 and pCGP454, are shown in Figure 2. A mixed probe of ^{32}P-labelled DNA fragments that included the coding regions of these five cytochrome P450 homologues (see Figures 2A and B) was used to screen 50,000 recombinants from cDNA library #1 for related sequences. A total of 152 hybridizing clones were detected under low stringency hybridization and washing conditions. A further 13 different cytochrome P450 homologues were identified by sequence analysis of DNA isolated from the hybridizing clones.

One of these clones, designated pCGP174, was shown to correspond to the Hf1 locus of Petunia (see International Patent Application No. PCT/AU92/00334). The nucleotide sequence of a full-length version of this clone, pCGP602, isolated from cDNA library #2 is shown in Figure 3. Ten of the thirteen other cytochrome P450 homologues isolated in the screen, pCGP161, pCGP162, pCGP163, pCGP165, pCGP166, pCGP167, pCGP168, pCGP169, pCGP171 and pCGP173 were used as a mixed probe to screen cDNA library #2 for further cytochrome P450 homologues (see next section).

Isolation of the cytochrome P450 homologue pCGP619 from *Petunia*

A mixed probe of ^{32}P-labelled cDNA inserts from pCGP161, pCGP162, pCGP163, pCGP165, pCGP166, pCGP167, pCGP168, pCGP169, pCGP171 and pCGP173 (Figure 4) was used to screen 1.5 x 10^5 recombinants from cDNA library #2. Over 200 hybridizing clones were detected with low stringency hybridization and washing in 2 x SSC and 1% SDS, at 65°C. Twenty-five of these clones hybridized to probes that included the internal HincII-EcoRV and EcoRV-HindIII fragments of

pCGP602 (Figure 3) under low stringency conditions, but not under high stringency conditions. Sequence analysis of this group of clones revealed that seventeen were siblings of pCGP602 (shown previously to correspond to the Hf1 locus of petunia - International Patent Application No. PCT/AU92/00334) and six were siblings of another petunia cDNA clone encoded by the Hf2 locus (International Patent Application No. PCT/AU92/00334). One clone showed no sequence homology to cytochromes P450, and one, designated pCGP619, showed 57% and 39% sequence homology to pCGP602 at the nucleotide and amino acid levels, respectively. The complete nucleotide sequence and deduced amino acid sequence of the pCGP619 cDNA are shown in Figure 5, and the restriction map outlining the sequencing strategy is shown in Figure 6.

Expression of pCGP619 cDNA in yeast

The cDNA insert from pCGP619 was ligated in a sense orientation behind the glyceraldehyde-3-phosphate dehydrogenase promoter in the yeast vector pYHCC101. The resulting construct, designated pCGP621, was then transformed into the yeast strain G-1315 (Ashikari *et al*, 1989). 3'-Hydroxylase activity was detected in extracts of G-1315/pCGP621, but not in extracts of the non-transgenic yeast (Figure 7). From this it was concluded that the cDNA insert from pCGP619 encoded a 3'-hydroxylase.

Expression of a 3'-hydroxylase cDNA in *Petunia*

The binary plasmid construct designated pCGP809 was introduced into the F_1 petunia hybrid Skr4 x Sw63 using Agrobacterium-mediated gene transfer. Leaf discs of Skr4 x Sw63 were co-cultivated with AGL0/pCGP809 and integration of the pCGP619 cDNA insert in the Skr4 x Sw63 genome was confirmed by Southern analysis of plants obtained after kanamycin selection.

The expression of the introduced 3'-hydroxylase cDNA in the Skr4 x Sw63 hybrid had a noticeable effect on flower colour. In parts of the petals of Skr4 x Sw63 the colour changed from light pink to red. The colour change observed may be described in terms of the numbers from the Royal Horticultural Society's Colour

Chart as having shifted from 55D-56C/D to 54A-55A. Other biochemical and physiological conditions will affect the individual outcome and the citing of the specific colour change achieved by expression of the 3'-hydroxylase cDNA in transgenic plants should not be interpreted as limiting the possible range of colour changes which may be observed.

Generation of mutants and derivatives of flavonoid 3'-hydroxylase

Using standard mutagenic techniques as hereinbefore disclosed, a range of mutants, derivatives and parts of flavonoid 3'-hydroxylase are obtainable, which may be useful in accordance with the present invention. For specific descriptions and protocols for such mutagenic techniques reference can conveniently be made to Sambrook *et al.* (1989). Examples of mutants, derivatives and parts of 3'-hydroxylase which are isolatable and contemplated herein include the following:

```
5'   GCT AAA GAG TTT AAG GAA    3'
     Ala Lys Glu Phe Lys Glu

5'   AAG AAA CTT CCA CCA GGT CCA TTT    3'
     Lys Lys Leu Pro Pro Gly Pro Phe

5'   TTG GAG TGG GCA ATG GC    3'
     Leu Glu Trp Ala Met Ala

5'   G GAC TTG TTT GCA GCA GG    3'
       Asp Leu Phe Ala Ala Gly

5'   CCA TTT GGT GCT GGT CGA AGA ATT TGC CCT GG    3'
     Pro Phe Gly Ala Gly Arg Arg Ile Cys Pro Gly
```

Detection of related sequences in plant species other than *Petunia*

Using standard Southern analysis techniques, a "nursery blot" was prepared of DNA isolated from a variety of plant species, including apple, carnation, cornflower, morning glory and rose to screen for genetic sequences related to the *petunia* 3'-hydroxylase. Results clearly showed the presence of related genetic

sequences in all the plants tested. The nursery blot comprised lanes 1 - 5 containing approximately 10 mg DNA from each of the above-mentioned species, respectively. The probe DNA used was the HindIII-EcoRV fragment from pCGP619. Southern analysis was conducted over a range of stringency conditions. Suitable stringency conditions indicating the presence of a number of similar sequences in each species were overnight incubation in 50% formamide, 1M NaCl, 1% SDS, 10% dextran sulphate at 42°C, followed by 3 x 30 min washes in 2 x SSC, 1% SDS at 60°C.

Isolation of a cytochrome P450-homologous PCR product from *Rosa*

Double-stranded rose petal cDNA, synthesized as described in Materials and Methods, was used as the template for amplification of sequences related to the petunia 3'-hydroxylase using oligonucleotides 7 and 190. A PCR product of approximately 400 bp was ligated into pBluescript and one of the recombinant plasmids recovered was designated pCGP635. The nucleotide sequence and deduced amino acid sequence of the pCGP635 insert are shown in Figure 8. This insert shows 60% similarity at the nucleotide level to the *Petunia* pCGP619 cDNA.

Isolation of cytochrome P450-homologous PCR products from *Dianthus*

Single-stranded carnation petal cDNA synthesized as described in Materials and Methods, was used as the template for amplification of sequences related to the petunia 3'-hydroxylase using oligonucleotides 7 and 190. A PCR product of approximately 400 bp was ligated into pBluescript. Sequence analysis of the recombinant plasmids revealed that two different cytochrome P450 homologues had been amplified and cloned. Representative clones of these two molecules were designated pCGP772 and pCGP773. The nucleotide sequence and deduced amino acid sequence of each insert are shown in Figures 9 and 10, respectively. Comparison of the deduced amino acid sequences with that of other cytochrome P450s yielded the following results:

	pCGP772	pCGP773
pCGP619	59.2%	64.8%

pCGP158 (Haem binding area)	62.9%	61.1%
pCGP168 (Haem binding area)	59.5%	
Avocado cytochrome P450		57.8%

Isolation of a cytochrome P450-homologous PCR product from *Chrysanthemum*

Chrysanthemum genomic DNA isolated as described in the Materials and Methods was used as the template for amplification of sequences related to the petunia 3'-hydroxylase using oligonucleotides 7 and 190. A PCR product of approximately 400 bp was ligated into the ddT-tailed pBluescript and one of the recombinant plasmids recovered was designated pCGP854. The nucleotide sequence and deduced amino acid sequence of 120 of these base pairs are shown in Figure 11. This sequence was compared with that from the *Petunia* cDNA clone pCGP619, shown in Figure 5, and shows 73% and 65% similarity at the DNA and amino acid level, respectively, to the segment of sequence between positions 971 and 1091.

Isolation of a *Chrysanthemum* petal cDNA clone with sequence similarity to the *Petunia* 3'-hydroxylase

The cDNA insert from pCGP619 was used to screen cDNA library #3 for related sequences. Using the hybridization and washing conditions described in the Materials and Methods, 64 hybridizing clones were detected. Twelve of these clones also hybridized to the insert from pCGP854. Sequence analysis of a putative full-length clone that hybridized to both the pCGP619 and pCGP854 probes revealed that it included an identical sequence to that of the PCR product sequence shown in Figure 11 and therefore encodes a putative chrysanthemum 3'-hydroxylase.

Expression of a *Chrysanthemum* petal cDNA clone in yeast

The petal cDNA clone can be ligated in a sense orientation behind the glyceraldehyde-3-phosphate dehydrogenase promoter in the yeast vector pYHCC101. The resulting construct is then transformed into the yeast strain G-1315 (Ashikari *et al*, 1989). Activity of the 3'-hydroxylase can be detected in extracts of G-1315 plus construct, but not in extracts of non-transgenic yeast. From

this result it can be concluded that the cDNA insert encodes a 3'-hydroxylase.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS:

1. A nucleic acid isolate comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, flavonoid 3'-hydroxylase enzyme or a functional derivative of the enzyme.

2. A nucleic acid isolate according to claim 1 wherein said nucleic acid is DNA or cDNA.

3. A nucleic acid isolate according to claim 1 wherein said nucleic acid is genomic DNA.

4. A nucleic acid isolate according to claim 1 or 2 or 3 wherein the enzyme is of petunia, carnation, chrysanthemum, rose, maize, snapdragon, tobacco, cornflower, pelargonium or morning glory origin.

5. A nucleic acid isolate according to claim 4 wherein the enzyme is of petunia, carnation, chrysanthemum or rose origin.

6. A nucleic acid isolate according to claim 4 wherein the enzyme is of petunia origin.

7. A nucleic acid isolate according to claim 1 having a nucleotide sequence comprising substantially all or part of the nucleotide sequence set forth in Figure 5 or having at least 40% similarity to one or more regions thereof.

8. A nucleic acid isolate according to any one of claims 1 to 7 when present in a transgenic plant.

9. A nucleic acid isolate according to claim 8 wherein the transgenic plant exhibits an altered level of flavonoid 3'-hydroxylase activity.

10. A nucleic acid isolate according to claim 9 wherein the altered level of flavonoid 3'-hydroxylase activity is a reduction in activity.

11. A nucleic acid isolate according to claims 8 or 9 wherein the transgenic plant is petunia, carnation, chrysanthemum, rose, snapdragon, tobacco, cornflower, pelargonium, lisianthus or morning glory.

12. A nucleic acid isolate according to claims 8 or 9 wherein the transgenic plant is petunia, carnation, chrysanthemum or rose.

13. A nucleic acid isolate according to claim 10 wherein the transgenic plant is a petunia.

14. A nucleic acid isolate according to any one of claims 1 to 13 contained in a vector.

15. A nucleic acid isolate according to claim 14 wherein the vector is a binary vector.

16. A nucleic acid isolate according to claim 14 wherein the vector is a viral vector.

17. A nucleic acid isolate according to claim 14 or 15 wherein the vector and nucleic acid isolate is pCGP809.

18. An oligonucleotide comprising a sequence of nucleotides capable of hybridizing to the nucleic acid isolate of claim 1.

19. An oligonucleotide according to claim 18 wherein the oligonucleotide is capable of hybridizing to the 5' region of the nucleic acid isolate according to claim 1.

20. An oligonucleotide according to claim 18 wherein the oligonucleotide is capable of hybridizing to the 3' region of the nucleic acid isolate according to claim 1.

21. An oligonucleotide according to any of claims 18, 19 and 20 selected from the list comprising:

GCTAAAGAGTTTAAGGAA

AAGAAACTTCCACCAGGTCCATTT

TTGGAGTGGGCAATGGC

GGACTTGTTTGCAGCAGG

CCATTTGGTGCTGGTCGAAGAATTTGCCCTGG

22. A flavonoid 3'-hydroxylase, or a functional derivative thereof, encoded by the nucleic acid according to claim 1.

23. A flavonoid 3'-hydroxylase according to claim 22 wherein said enzyme is of petunia, carnation, chrysanthemum, rose, maize, snapdragon, tobacco, cornflower, pelargonium or morning glory origin.

24. A flavonoid 3'-hydroxylase according to claim 22 wherein said enzyme is of petunia, carnation, chrysanthemum or rose origin.

25. A flavonoid 3'-hydroxylase according to claim 23 wherein the enzyme is of petunia origin.

26. A flavonoid 3'-hydroxylase according to claim 23 or 24 or 25 having an amino acid sequence comprising substantially all or a part of the amino acid sequence set forth in Figure 5 or having at least 40% similarity to one or more regions thereof.

27. A flavonoid 3'-hydroxylase according to any one of claims 22 to 26 when produced or capable of being produced in a transgenic plant.

28. A flavonoid 3'-hydroxylase according to claim 27 when the transgenic plant is petunia, carnation, chrysanthemum, rose, snapdragon, tobacco, cornflower, pelargonium, lisianthus or morning glory.

29. A flavonoid 3'-hydroxylase according to claim 27 when the transgenic plant is petunia, carnation, chrysanthemum or rose.

30. A flavonoid 3'-hydroxylase according to claim 27 wherein the transgenic plant is a petunia.

31. A method for producing a transgenic plant capable of synthesizing a 3'-hydroxylase or active mutants or derivatives thereof, said method comprising stably transforming a cell of a suitable plant with the nucleic acid according to claim 1 or any one of claims 14 to 16 under conditions permitting the eventual expression of said nucleic acid, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid.

32. A method according to claim 31 wherein the expression of the nucleic acid is developmentally regulated.

33. A method according to claim 32 wherein the enzyme is of petunia, carnation, chrysanthemum, rose, maize, snapdragon, tobacco, cornflower, pelargonium or morning glory origin.

34. A method according to claim 33 wherein the enzyme is of petunia, carnation, chrysanthemum or rose origin.

35. A method according to claim 34 wherein the enzyme is of petunia origin.

36. A method according to any one of claims 31 to 35 wherein the transgenic plant is petunia, carnation, chrysanthemum, rose, snapdragon, tobacco, cornflower, pelargonium, lisianthus or morning glory.

37. A method according to claim 36 wherein the transgenic plant is petunia, carnation, chrysanthemum or rose.

38. A method according to claim 36 wherein the transgenic plant is a petunia.

39. A method for producing a transgenic plant with reduced endogenous or existing flavonoid 3'-hydroxylase activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding said flavonoid 3'-hydroxylase, regenerating a transgenic plant from the cell and, where necessary, growing said transgenic plant under conditions sufficient to permit expression of the nucleic acid.

40. A method for producing a genetically modified plant with reduced endogenous or existing flavonoid 3'-hydroxylase activity, said method comprising altering the flavonoid 3'-hydroxylase gene through modification of the endogenous sequences via homologous recombination from an appropriately altered flavonoid 3'-hydroxylase gene or derivative or part thereof introduced into the plant cell and regenerating the genetically modified plant from the cell.

41. A method according to claim 39 wherein the expression of the nucleic acid molecule is developmentally regulated.

42. A method according to claim 39 wherein the nucleic acid molecule has a nucleotide sequence which is substantially complementary or at least 40% complementary to all or part of the nucleotide sequence set forth in Figure 5.

43. A method according to claims 39 or 40 wherein the transgenic plant or genetically altered plant is petunia, carnation, chrysanthemum, rose, snapdragon, tobacco, cornflower, pelargonium, lisianthus or morning glory.

44. A method according to claims 39 or 40 wherein the transgenic plant or genetically altered plant is petunia, carnation, chrysanthemum or rose.

45. A method according to claim 44 wherein the transgenic or genetically altered plant is a petunia.

46. A method according to claim 39 or 40 wherein the transgenic plant is produced by introducing the nucleic acid according to any one of claims 14 to 16.

47. A transgenic plant or progeny thereof carrying an introduced nucleic acid molecule comprising a nucleotide sequence encoding, or complementary to a sequence encoding, flavonoid 3'-hydroxylase enzyme, or a functional derivative thereof.

48. A transgenic plant according to claim 47 wherein the enzyme is of petunia, carnation, chrysanthemum, rose, maize, snapdragon, tobacco, cornflower, pelargonium or morning glory origin.

49. A transgenic plant according to claim 48 wherein the enzyme is of petunia, carnation, chrysanthemum or rose origin.

50. A transgenic plant according to claim 49 wherein the enzyme is of petunia origin.

51. A transgenic plant according to claim 48 or 49 or 50 wherein the enzyme has an amino acid sequence substantially as set forth in Figure 5 or having at least 40% similarity to one or more regions thereof.

52. A transgenic plant according to any one of claims 47 to 51 wherein said plant is petunia, carnation, chrysanthemum, rose, snapdragon, tobacco, cornflower, pelargonium, lisianthus or morning glory.

53. A transgenic plant according to claim 52 wherein said plant is petunia, carnation, chrysanthemum or rose.

54. A transgenic plant according to claim 53 wherein the transgenic plant is a petunia.

FIGURE 1A

FIGURE 1B

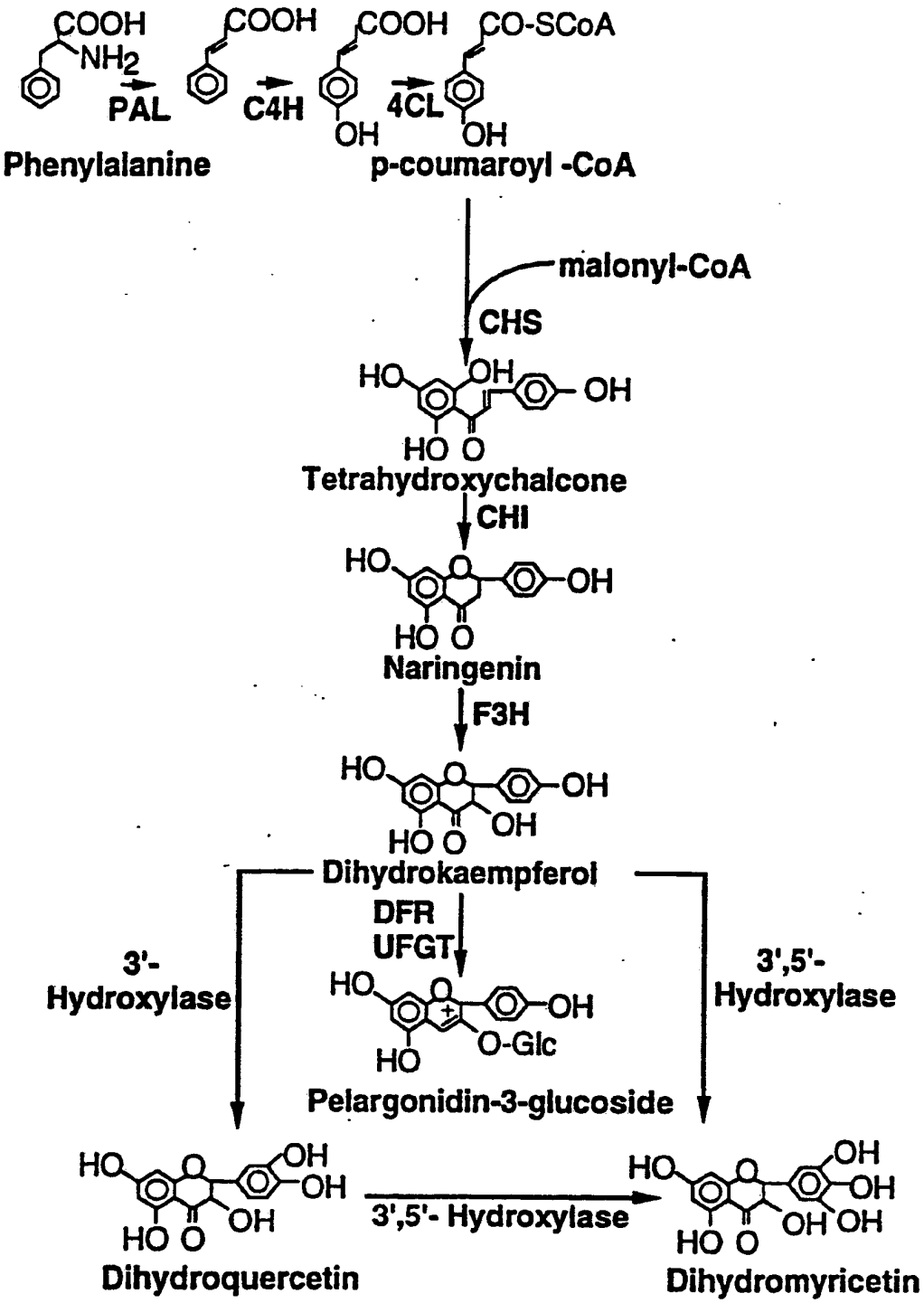

FIGURE 1A

SUBSTITUTE SHEET

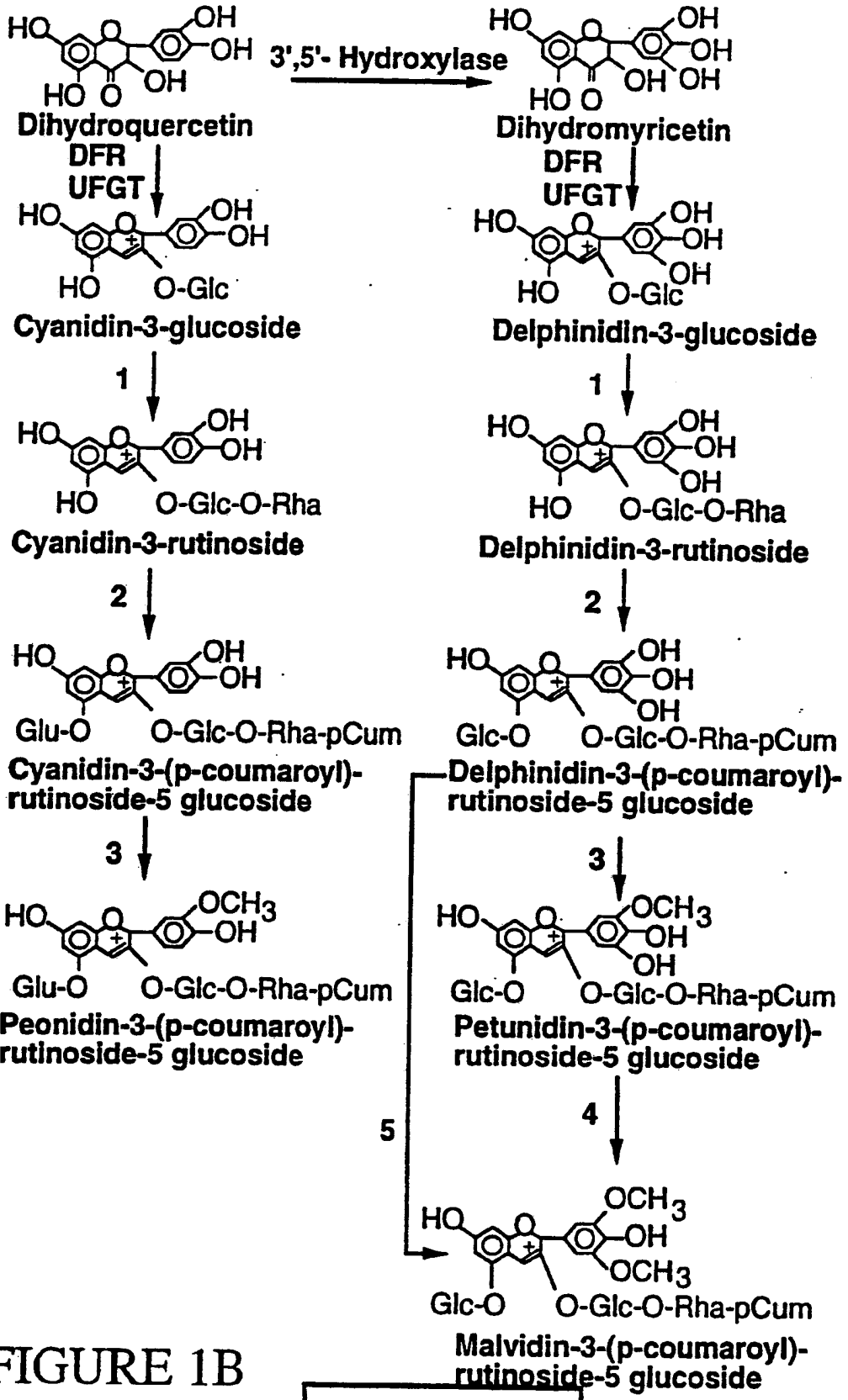
3',5'- Hydroxylase
Dihydroquercetin
Dihydromyricetin
DFR
UFGT
Cyanidin-3-glucoside
Delphinidin-3-glucoside
1
Cyanidin-3-rutinoside
Delphinidin-3-rutinoside
2
Glu-O
Glc-O
O-Glc-O-Rha-pCum
Cyanidin-3-(p-coumaroyl)-rutinoside-5 glucoside
Delphinidin-3-(p-coumaroyl)-rutinoside-5 glucoside
3
Peonidin-3-(p-coumaroyl)-rutinoside-5 glucoside
Petunidin-3-(p-coumaroyl)-rutinoside-5 glucoside
4
5
Malvidin-3-(p-coumaroyl)-rutinoside-5 glucoside

FIGURE 1B

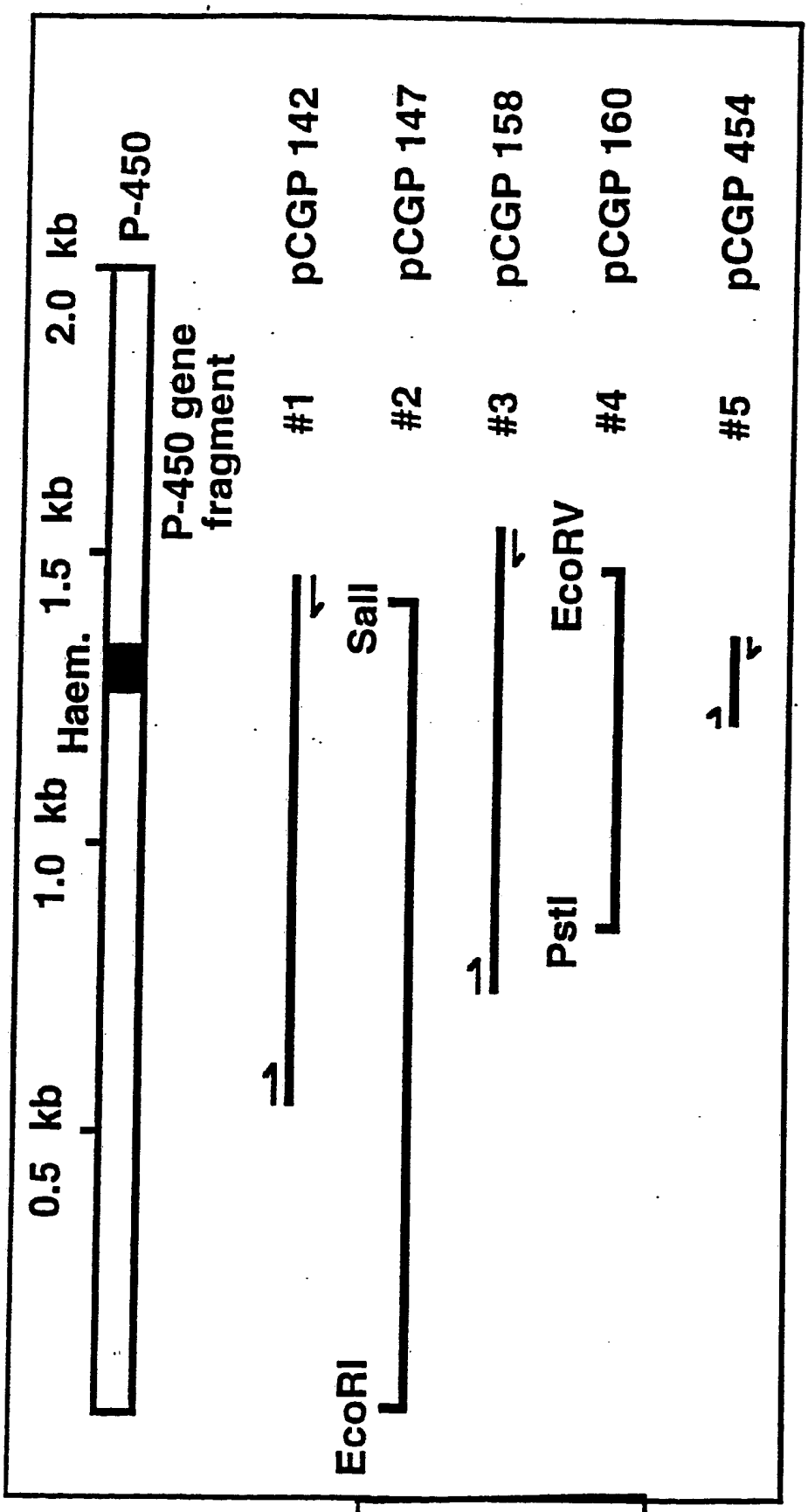
0.5 kb
1.0 kb
Haem.
1.5 kb
2.0 kb
P-450
P-450 gene fragment
pCGP 142
pCGP 147
pCGP 158
pCGP 160
pCGP 454
#1
#2
#3
#4
#5
SalI
EcoRV
PstI
EcoRI

FIGURE 2A

pCGP 142

FIGURE 2B

```
 F   S   S   I   R   N   D   E   I   S   S   L
TTT AGT TCA ATT CGG AAT GAT GAG ATT TCG AGT CTC

 I   S   S   I   H   S   M   N   G   S   V   V
ATT TCA TCA ATT CAT TCC ATG AAC GGT TCT GTT GTC

 N   M   T   Q   K   I   L   C   F   T   N   S
AAC ATG ACA CAA AAG ATT CTT TGT TTT ACA AAC TCT

 V   T   C   R   T   A   F   G   K   V   Y   K
GTG ACT TGT AGA ACA GCT TTC GGG AAA GTA TAC AAA

 N   Q   N   E   L   I   N   L   M   R   E   V
AAT CAA AAT GAA TTG ATA AAC TTG ATG AGG GAA GTA

 L   E   L   V   G   G   F   D   ---------------
CTG GAA TTA GTA GGA GGA TTT GAT ---------------

 F   E   N   S   P   V   E   F   I   G   N   H
TTT GAA AAT TCT CCG GTT GAG TTT ATT GGA AAT CAC

 F   E   L   V   P   F   G   A   G   K   R   I
TTT GAG CTT GTT CCG TTT GGT GCA GGA AAA AGG ATT

 C   P   G   M   Q   F   G   L   A   N   I   R
TGT CCA GGA ATG CAA TTT GGT TTA GCT AAT ATT AGA

 H   P   L   A   R   F   L   Y   H   F   N   W
CAT CCT TTG GCT CGA TTC CTC TAC CAT TTT AAC TGG

 A   L   P   Y   E   T   N   P   E   D   L   D
GCG CTT CCA TAT GAA ACT AAT CCT GAA GAT TTA GAT

 S   L   K   N   M   D
AGT CTG AAA AAT ATG GAT TAA GTGCAGCAAAAGAGAAAGA
TCTATACTTAATTGCCGTAGATCACAAAGAAGGTGATATATAAATTC
TGATGTTCTGCTTTAAATGGTGAAAGTCATACTCTACACAATGCTTC
ATCTCCTTAATTTGAGTTTGGTGTACATTTGTGTCTCCCTTTTAGCT
TTGAATTTCACCTTGAAAAATGATCACATTTTCTTTTTCTGTTACTC
CAATTAAGATATATGTTGTGGTTGGTCAATTATGCCATATTTATCAA
AAGATCAAATCAATTCCCTCGTTGATAAGTATAGATTATAAAACTGA
TTAATGAATCAAAAAAAAAAAAAAAA
```

FIGURE 2C

FIGURE 2D

FIGURE 2E

FIGURE 2C

pCGP147

```
    Q  F  F  N  L  V  S  F  L  L  I  V  F  S  L
TGCAATTTTTCAACTTGGTTTCCTTTCTCCTTATTGTATTTTCCCTC
         10         20         30         40

 I  S  L  R  K  W  K  K  S  N  C  Q  T  K  K  L
ATTTCATTAAGAAAATGGAAGAAATCCAATTGTCAAACCAAAAAATTG
         58         68         78         88

 P  P  G  P  W  K  V  P  F  L  G  S  L  L  H  M
CCTCCAGGCCCATGGAAAGTACCTTTTCTTGGAAGCTTGCTTCATATG
        106        116        126        136

 V  G  G  L  P  H  H  V  L  R  D  L  A  K  K  Y
GTAGGTGGACTTCCACACCATGTCCTTAGAGATTTAGCCAAAAAATAT
        154        164        174        184

 G  P  I  M  H  L  Q  L  G  K  I  S  A  V  V  V
GGACCAATTATGCACCTTCAACTAGGTAAAATTTCTGCCGTTGTAGTT
        202        212        222        232

 T  S  P  E  M  A  R  K  V  L  K  T  H  D  L  A
ACTTCTCCTGAGATGGCAAGAAAAGTACTAAAAACTCATGACCTTGCA
        250        260        270        280

 F  A  Y  R  P  K  L  L  G  I  E  I  V  C  Y  N
TTTGCATATAGGCCTAAACTTCTAGGCATTGAGATTGTCTGCTATAAT
        298        308        318        328

 S  S  D  I  A  F  S  P  Y  G  D  Y  W  R  Q  M
AGTTCAGACATTGCCTTTTCCCCGTATGGTGATTACTGGAGGCAAATG
        346        356        366        376

 R  K  I  C  V  L  E  V  L  S  A  K  N  V  R  S
CGTAAAATTTGTGTATTGGAAGTGCTTAGTGCCAAAAATGTCCGGTCA
        394        404        414        424

 F  N  S  I  R  R  D  E  I  L  L  M  I  D  F  L
TTTAACTCGATTAGACGAGATGAAATACTTCTTATGATCGATTTTTTG
        442        452        462        472

 R  S  S  S  G  K  P  V  N  I  T  E  R  I  F  S
CGATCATCTTCTGGTAAGCCAGTTAATATAACAGAAAGGATCTTTTCA
        490        500        510        520
```

FIGURE 2D

```
 F  T  S  S  M  I  C  R  S  V  F  G  K  R  I  K
TTCACAAGCTCTATGATTTGTAGATCAGTATTTGGGAAAAGAATAAAG
         538        548        558        568

 E  K  D  E  C  I  R  H  V  K  K  M  T  G  L  I
GAGAAAGACGAATGTATACGACATGTGAAAAAAATGACAGGCTTAATA
         586        596        606        616

 D  G  F  D  V  A  D  I  F  P  S  L  R  F  L  H
GATGGGTTCGATGTGGCTGACATATTCCCTTCGTTGAGGTTTCTTCAT
         634        644        654        664

 V  L  I  G  M  K  G  K  I  M  D  V  H  R  K  V
GTACTAATCGGTATGAAGGGTAAAATTATGGATGTTCATCGTAAGGTA
         682        692        702        712

 D  A  I  V  E  E  V  M  N  E  H  K  E  T  L  R
GATGCTATTGTTGAGGAAGTCATGAATGAGCACAAAGAAACTCTTCGA
         730        740        750        760

 T  G  K  T  N  G  E  V  G  G  E  D  L  I  D  V
ACTGGCAAGACCAATGGTGAAGTGGGAGGAGAAGATTTAATTGATGTA
         778        788        798        808

 L  L  R  L  K  E  E  G  D  L  Q  L  P  I  T  N
TTGCTAAGACTTAAGGAAGAGGGAGACCTTCAACTTCCAATCACAAAT
         826        836        846        856

 D  N  I  K  A  I  F  N  D  M  F  A  A  G  T  E
GACAACATCAAAGCCATTTTTAATGACATGTTTGCTGCGGGAACAGAA
         874        884        894        904

 T  S  S  T  T  I  N  W  A  M  V  E  L  M  K  N
ACTTCATCAACAACAATTAACTGGGCCATGGTAGAACTGATGAAAAAT
         922        932        942        952

 P  S  V  F  A  K  A  Q  A  E  V  R  E  V  F  K
CCAAGTGTATTCGCGAAAGCTCAAGCAGAGGTAAGAGAAGTCTTCAAA
         970        980        990       1000

 G  K  E  T  F  D  E  D  D  I  E  E  L  N  Y  L
GGGAAAGAAACTTTCGATGAAGATGATATCGAGGAGCTGAATTACCTT
        1018       1028       1038       1048

 K  L  V  I  R  E  T  L  R  L  H  P  P  L  P  L
AAGTTAGTCATTAGAGAAACTTTAAGACTCCACCCTCCACTTCCACTT
        1066       1076       1086       1096
```

```
 L  L  P  R  E  C  R  R  E  T  E  I  N  G  Y  T
TTGCTTCCAAGAGAATGTCGGAGAGAAACAGAAATAAATGGCTACACT
       1114       1124       1134       1144

 I  P  L  N  T  K  V  I  V  N  V  W  A  I  G  R
ATTCCTTTAAATACCAAAGTCATAGTTAATGTTTGGGCTATTGGAAGA
       1162       1172       1182       1192

 D  P  K  Y  W  D  D  A  E  S  F  K  P  E  R  F
GATCCAAAATATTGGGATGATGCAGAAAGCTTTAAGCCTGAGAGATTT
       1210       1220       1230       1240

 E  H  N  S  L  N  F  A  G  N  N  F  E  Y  L  P
GAACATAACTCTTTGAATTTTGCTGGCAATAATTTTGAATATCTTCCT
       1258       1268       1278       1288

 F  G  S  G  R  R  I  C  P  G  I  S  F  G  L  A
TTTGGTAGTGGAAGGAGGATTTGCCCCGGAATATCATTTGGTTTAGCT
       1306       1316       1326       1336

 N  V  Y  H  P  L  A  Q  L  L  Y  H  F  D  W  R
AATGTTTATCATCCATTGGCTCAATTGTTGTATCATTTCGATTGGAGA
       1354       1364       1374       1384

 L  P  T  G  V  D  P  N  D  F  E  L  T  S  *
CTTCCTACTGGGGTCGACCCAAATGACTTTGAATTGACTAGTTAGCTG
       1402       1412       1422       1432

GAGTAACTACTGGTAGGAAAAGAGACCTTTACTTGATTTTCACTCCTT
       1450       1460       1470       1480

ATTCACCTTCTCTAAAGTGATTAAATGG-GCAAATTTTAATTTGAAAT
       1498       1508       1518       1528

AATACTTTTTCTTGTTTACATTTCTCTCCCATTGTTGTATTTCATTTA
       1546       1556       1566       1576

CCTATTGTTGTACTTCTTTCTTTTGTTGATGTCTTAGGTTTTACCTAT
       1594       1604       1614       1624

TTCTATGCATTTGTATTTAAAAAAAAAAAAAAAA
       1642       1652       1662
```

FIGURE 2E

FIGURE 2F

pCGP158

```
Gly Met Met Lys Gln Gly Asp Phe Leu Asp Val Leu
GGG ATG ATG AAG CAA GGA GAT TTC TTG GAT GTA CTT
          ------------------------>
Leu Asp Gln Cys Asp Glu Glu Gly Ser Gly Phe Asp
CTT GAT CAA TGT GAT GAA GAA GGG TCT GGA TTT GAT

Arg Gln Thr Ile Lys Pro Leu Ile Leu Asp Leu Phe
CGC CAA ACT ATC AAG CCT CTC ATC CTG GAT TTA TTC

Ile Ala Gly Ser Asp Thr Ser Ala Ile Thr Thr Glu
ATT GCT GGA AGT GAT ACA TCT GCC ATA ACA ACA GAA

Trp Ala Met Ala Glu Leu Leu Arg Lys Pro Gln Glu
TGG GCA ATG GCA GAA CTA CTT CGA AAA CCT CAA GAA

-------------------------------------------------
-------------------------------------------------

Phe Val Asn Ala Trp Ala Ile Gly Arg Asp Pro Lys
TTT GTG AAT GCA TGG GCA ATT GGA AGA GAT CCA AAA

Tyr Trp Glu Lys Pro Leu Glu Phe Met Pro Glu Arg
TAC TGG GAA AAA CCA CTG GAG TTT ATG CCT GAA AGA

Phe Leu Lys Cys Ser Leu Asp Tyr Lys Gly Arg ---
TTC TTG AAG TGT AGT TTG GAT TAC AAA GGT AGG G--

Phe Glu Tyr Ile Pro Phe Gly Ala Gly Arg Arg Ile
TTT GAG TAT ATA CCA TTT GGC GCA GGT CGA AGA ATT

Cys Pro Gly Met Pro His Cys Asn Lys Asp Gly Glu
TGT CCT GGA ATG CCA CAT TGC AAT AAG GAT GGT GAA

Phe Asp Ala Gly Phe Asp Tyr Ser Pro Phe Ser Trp
TTT GAT GCT GGC TTC GAT TAT TCA CCA TTT AGT TGG

Glu Leu Pro --- Gly Met Ala Pro Lys --- Leu Asn
GAA TTA CCT -AA GGA ATG GCA CCA AAG -AT TTG AAC

Met Glu Glu Gln Phe Gly Val Thr Leu Arg Lys Ala
ATG GAG GAA CAG TTT GGA GTT ACC TTG AGG AAG GCT

Ile Pro Leu Ile Ala Ile Pro Ser Met Glu Glu Lys
ATT CCC CTT ATT GCC ATT CCC AGT ATG GAA GAA AAG

Val Ile Phe
GTC ATA TTT TAG CCCAAAAGCTATGCATTTTGTGTGTATGTTT
          <------------------------
```

FIGURE 2G

pCGP 160

```
 K   Q   I   N   A   L   L   V   E   I   F   G
AAA CAG ATC AAT GCA TTG CTT GTG GAA ATA TTT GGA

 A   G   T   E   S   T   T   A   T   S   Q   W
GCT GGT ACA GAA TCT ACA ACT GCT ACA AGC CAA TGG

 M   L   V   E   L   L   R   N   R   Q   A   L
ATG CTT GTA GAA CTC CTT AGA AAT CGA CAA GCC TTG

---------- P   K   D   T   Q   V   M   V   N
----------CCC AAA GAC ACT CAA GTT ATG GTA AAC

 E   W   A   I   A   Y   D   P   K   I   W   G
GAG TGG GCG ATT GCG TAT GAT CCT AAG ATT TGG GGC

 S   F   K   P   Q   R   F   I   D   S   K   I
AGC TTC AAA CCC GAA AGG TTT ATC GAT TCA AAA ATA

 D   P   L   D   H   K   G   Q   N   F   E   Y
GAT CCT TTG GAC CAC AAA GGG CAA AAT TTT GAA TAT

 F   P   F   G   S   G   R   R   I   C   A   G
TTT CCT TTT GGT TCT GGA AGG AGA ATT TGT GCT GGA

 E   P   L   A   S   R   V   I   P   L   A   V
GAA CCT TTG GCT TCT AGG GTT ATT CCC TTA GCT GTT

 A   S   M   I   H   K   F --------
GCT TCT ATG ATC CAT AAG TTT --------GATATCACTAT

GTTAGAAGATCCACTCTCATCATTCCTAAGTTGAGAAGAGTGAGGAA

ATTAAAAGAAGCAGAAGATATGTTACTATAAAAACTCGTTATATATA

TATATATTGCTGTATCTATATATGTGTGAATGATCTGCTGCTCATGT

TGTGTTTTGTTGTTTGTGTACTATAGGTCATACCTAAGTTGATGAAA

TGTCTCTGAGAATATATACTCCTTATATAATAGGAGTAATTTACCGA

TAATTAATATTCCTGCGACAAAAAAAAAAAAAAAA
```

pCGP454

```
 -   R   E   S   M   E   D   V   R   L   L   G
CT CGA GAA TCA ATG GAA GAT GTA AGA TTA CTA GGC

 Y   H   I   P   A   K   T   R   L   F   I   N
TAT CAC ATA CCT GCT AAA ACG AGA CTC TTT ATC AAT

 A   W   T   M   G   R   D   P   L   T   W   E
GCT TGG ACA ATG GGG AGA GAC CCA CTA ACA TGG GAA

 N   P   E   E   Y   Q   P   E   R   F   L   N
AAT CCA GAA GAG TAT CAG CCA GAG AGA TTC TTG AAT

 R   D   T   D   V   K   G   V   N   F   E   F
AGA GAT ACT GAT GTC AAA GGA GTA AAC TTT GAG TTC

 I   P   F   G   A   G   R   S
ATT CCC TTT GGC GCC GGC AGA AGC
```

FIGURE 2H

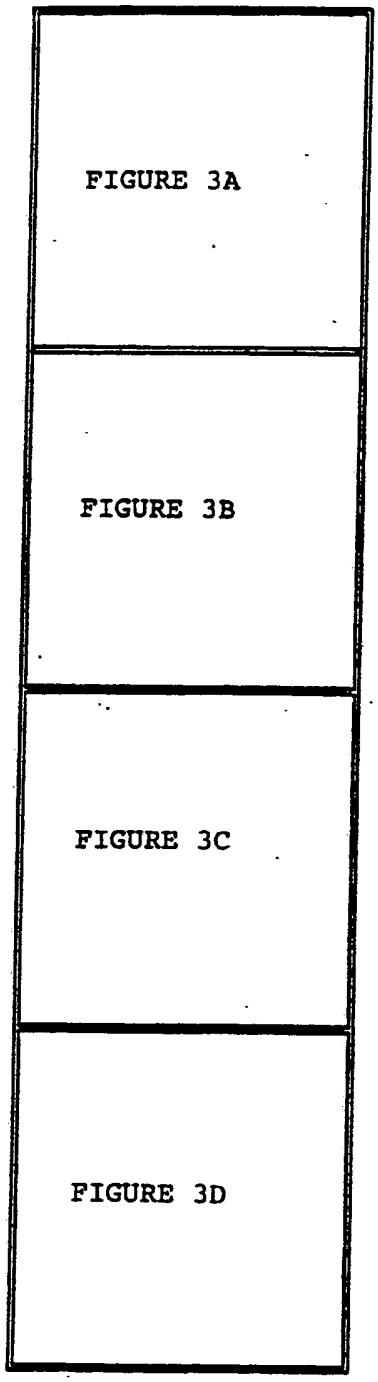

FIGURE 3A

pCGP 602

```
CTTTCTACTAGCTACTTCGTTATATATATGTAAAATTGTGACTTT
        10        20        30        40

GAAAATCATTTAAATTATCATAAGGTTCATTTTATCTTGATCAAA
        55        65        75        85

                                          M  M  L
ATATTTACTTCGGCCATATACGTTTTCCTTTAGTCATGATGCTAC
       100       110       120       130

L  T  E  L  G  A  A  T  S  I  F  L  I  A  H
TTACTGAGCTTGGTGCAGCAACTTCAATCTTTCTAATAGCACACA
       145       155       165       175

I  I  I  S  T  L  I  S  K  T  T  G  R  H  L
TAATCATTTCAACTCTTATTTCAAAAACTACCGGCCGGCATCTAC
       190       200       210       220

P  P  G  P  R  G  W  P  V  I  G  A  L  P  L
CGCCGGGGCCAAGAGGGTGGCCGGTGATCGGAGCACTTCCACTTT
       235       245       255       265

L  G  A  M  P  H  V  S  L  A  K  M  A  K  K
TAGGAGCCATGCCACATGTTTCCTTAGCTAAAATGGCAAAAAAAT
       280       290       300       310

Y  G  A  I  M  Y  L  K  V  G  T  C  G  M  A
ATGGAGCAATCATGTATCTCAAAGTTGGAACATGTGGCATGGCAG
       325       335       345       355

V  A  S  T  P  D  A  A  K  A  F  L  K  T  L
TTGCTTCTACCCCTGATGCTGCTAAAGCATTCTTGAAAACACTTG
       370       380       390       400

D  I  N  F  S  N  R  P  P  N  A  G  A  T  H
ATATCAACTTCTCCAATCGTCCACCTAATGCAGGTGCCACTCACT
       415       425       435       445

L  A  Y  N  A  Q  D  M  V  F  A  H  Y  G  P
TAGCTTATAATGCTCAAGACATGGTTTTTGCACATTATGGACCAC
       460       470       480       490

R  W  K  L  L  R  K  L  S  N  L  H  M  L  G
GATGGAAGTTGCTAAGGAAATTAAGCAACTTGCATATGCTAGGGG
       505       515       525       535
```

FIGURE 3B

```
G  K  A  L  E  N  W  A  N  V  R  A  N  E  L
GAAAAGCCTTAGAGAATTGGGCAAATGTTCGTGCCAATGAGCTAG
        550        560        570        580

G  H  M  L  K  S  M  S  D  M  S  R  E  G  Q
GGCACATGCTAAAATCAATGTCCGATATGAGTCGAGAGGGCCAGA
        595        605        615        625

R  V  V  V  A  E  M  L  T  F  A  M  A  N  M
GGGTTGTGGTGGCGGAGATGTTGACATTTGCCATGGCCAATATGA
        640        650        660        670

I  G  Q  V  M  L  S  K  R  V  F  V  D  K  G
TCGGACAAGTGATGCTAAGCAAAAGAGTATTTGTAGATAAAGGTG
        685        695        705        715

V  E  V  N  E  F  K  D  M  V  V  E  L  M  T
TTGAGGTAAATGAATTTAAGGACATGGTTGTAGAGTTAATGACAA
        730        740        750        760

I  A  G  Y  F  N  I  G  D  F  I  P  C  L  A
TAGCAGGGTATTTCAACATTGGTGATTTTATTCCTTGTTTAGCTT
        775        785        795        805

W  M  D  L  Q  G  I  E  K  R  M  K  R  L  H
GGATGGATTTACAAGGGATAGAAAAACGAATGAAACGTTTACATA
        820        830        840        850

K  K  F  D  A  L  L  T  K  M  F  D  E  H  K
AGAAGTTTGATGCTTTATTGACAAAGATGTTTGATGAACACAAAG
        865        875        885        895

A  T  T  Y  E  R  K  G  K  P  D  F  L  D  V
CAACTACCTATGAACGTAAGGGGAAACCAGATTTTCTTGATGTTG
        910        920        930        940

V  M  E  N  G  D  N  S  E  G  E  R  L  S  T
TTATGGAAAATGGGGACAATTCTGAAGGAGAAAGACTCAGTACAA
        955        965        975        985

T  N  I  K  A  L  L  L  N  L  F  T  A  G  T
CCAACATCAAAGCACTTTTGCTGAATTTGTTCACAGCTGGTACGG
       1000       1010       1020       1030
```

FIGURE 3C

```
D  T  S  S  S  A  I  E  W  A  L  A  E  M  M
ACACTTCTTCTAGTGCAATAGAATGGGCACTTGCAGAAATGATGA
      1045       1055       1065       1075

K  N  P  A  I  L  K  K  A  Q  A  E  M  D  Q
AGAACCCTGCCATTTTGAAAAAAGCACAAGCAGAAATGGATCAAG
      1090       1100       1110       1120

V  I  G  R  N  R  R  L  L  E  S  D  I  P  N
TCATTGGAAGAAATAGGCGTTTACTCGAATCCGATATCCCAAATC
      1135       1145       1155       1165

L  P  Y  L  R  A  I  C  K  E  T  F  R  K  H
TCCCTTACCTCCGAGCAATTTGCAAAGAAACATTTCGAAAACACC
      1180       1190       1200       1210

P  S  T  P  L  N  L  P  R  I  S  N  E  P  C
CTTCTACACCATTAAATCTTCCTAGGATCTCGAACGAACCATGCA
      1225       1235       1245       1255

I  V  D  G  Y  Y  I  P  K  N  T  R  L  S  V
TAGTCGATGGTTATTACATACCAAAAAACACTAGGCTTAGTGTTA
      1270       1280       1290       1300

N  I  W  A  I  G  R  D  P  Q  V  W  E  N  P
ACATATGGGCAATTGGAAGAGATCCCCAAGTTTGGGAAAATCCAC
      1315       1325       1335       1345

L  E  F  N  P  E  R  F  L  S  G  R  N  S  K
TAGAGTTTAATCCCGAAAGATTCTTGAGTGGAAGAAACTCCAAGA
      1360       1370       1380       1390

I  D  P  R  G  N  D  F  E  L  I  P  F  G  A
TTGATCCTCGAGGGAACGATTTTGAATTGATACCATTTGGTGCTG
      1405       1415       1425       1435

G  R  R  I  C  A  G  T  R  M  G  I  V  M  V
GACGAAGAATTTGTGCAGGAACAAGAATGGGAATTGTAATGGTGG
      1450       1460       1470       1480

E  Y  I  L  G  T  L  V  H  S  F  D  W  K  L
AATATATATTAGGAACTTTGGTTCATTCATTTGATTGGAAATTAC
      1495       1505       1515       1525
```

```
P  S  E  V  I  E  L  N  M  E  E  A  F  G  L
CAAGTGAAGTTATTGAGTTGAATATGGAAGAAGCTTTTGGCTTAG
      1540       1550       1560       1570

A  L  Q  K  A  V  P  L  E  A  M  V  T  P  R
CTTTGCAGAAAGCTGTCCCTCTTGAAGCTATGGTTACTCCAAGGT
      1585       1595       1605       1615

L  Q  L  D  V  Y  V  P  *
TACAATTGGATGTTTATGTACCATAGCTATAGATGTGTATTGTGC
      1630       1640       1650       1660

TATAATTGCGCATGTTGTTGGTTGTAGCATGAGATATTAAAAGGA
      1675       1685       1695       1705

GTACATGAAGCGCATTGCATGAGTTTAACTTGTAGCTCCTTAATA
      1720       1730       1740       1750

TTTTAGGTATTTTTCAATTAATAAGTTCTTGTTGGTTGGGTAAAA
      1765       1775       1785       1795

AAAAAAAAAAAA
      1810
```

FIGURE 3D

FIGURE 4A

1)pCGP161

CCAGACACCCACAAACTTCCATACCTTCAGGCTGTGATCAAGGAGACTCT
TCGTCTCCGGATGGCAATTCCTCTATTAGTCCCACACATG----------
----------AAACTTTACAGAAAACGTTCATCTTTTT
ATGTCATATCAAGTCTTCTTGGACTGGTTCGTTATTACACCTACCTATCT
GAATGTATTTTT

2)pCGP162

ACGAACATGGGAAAATCCAGAAGAGTATCAGCCAGAGAGATTCTTGAATA
GTGATATTGATGTCAAAGGACTAAACTTTGAGTTGATTCC----------
---ATGGCTAGTAGCTACTTCTTTCATGATATCTGTAATAAGTGTAGTGC
TCGACTCCTTCAGGCGAGTTGTGTGTTTAATTTCTCCAGTATC

3)pCGP163

AAGTTCTTTCCATCAGTTATCAAACAAACCATGAGGCTGCATCCCCCTCT
CCCTTTATTACTATTAAGGGAAAGCAAGGAATCTTGTGAA----------
--------GATAGGGAGCGGTTTACTCCCTTCGTGGCCTTACCATTACACT
AACAATGAATGGGCTTGGAAATAGTCTCAGATGTTTTTAAAGAAAAC

4)pCGP165

GTGATTTTCCAAAAGAATCTCACTTGCTGCAGATGTCCTATGTTCAAGCC
TGTGTGAAGGAAACTCTTAGGTTGCATCCTCCGGCGCCAT----------
-----TATTTATTGAAGTTGAGAAACTTATGTATGAAAGTGTCATACAGA
ACTACTGCCCATGTGGTGTGTTTTAGTACTTCTTTTTTTTGGGT

5)pCGP166

ATGTCCTATGTTCAAGCCTGTGTGCCGGAAACTCTTAGGTTGCATCCTCC
GGCGCCATTGCTACTTCCACATGCGTGCAATCGAAACATG----------
----------TCCCCCTGGATTTGTACACTAGATACAAGACTT
AGCGGTCCTGGTGTAATCTCAATTCTCATGTGGTTATAAACAGAAGTTCT
TCTGGTG

6)pCGP167

GAAACATTATCAATGAACATGTTAAGAATCGAGCACTCGGAAGCAAGGGA
AATGGTGCGTTTGGAGGTGAAGATTTGGTTGATGTTTTAC----------
--------GGTTGGGGTAAATTGGGGCCCCCCTTTTAAGGCTTTGGAATTT
CCACCTGGAAAAATGGACCCCATTTTCCTTTTCCTGTACCTCCAATT

7)pCGP168

AAAACTGCAAACTAGCGATAACGCTGATGTTCTTGATGTGTTGTTGCATA
CTAGCGAGGAAGATCCAGAGGCAATCGACAGAATTCACAT--------

8)pCGP169

CAGTACACTCTTTGTGTTCATCATATCTCTTCACATTGCTCACAAGCTCG
ACCATGGCCGTCGGTAAGAACAAGAGGATTTCCAAAGGCA----------
-----AATACCACATTCTGATGATTCACTTGATATATGTGTACCTTTATG
TCATTTAATGGCACAACAATTCTGGGGACTTAGGTTCAAAGAAGC

9)pCGP171

CTGTAGGGTTACCGTTCATTGGAAATTTGCATCAATATGATACTTTAAAG
CCGCATATCTACTTCTGGAAACTTTCTAGGAAGTATGGAA----------
-----TACTTTCGGTTTTGAATTATGTATACATATATAAAACAAATGTGA
AATGTATACATATAATAAAATTGCTCTCATGATATACTTCTCTAT

10)pCGP173

TTCTGGAAATGTTTCTAGCTGGTACAGAGACATCTAGCAGCACAACAGAG
TGGGCACTAACTGAACTCCTTCGAAACCCAGAAACAATGG----------
-----ACAATTCTTACGCTGAATTTGTTGTTCGCCCTTTTATTTTCAGTT
TGATTGTATCCAAAGGATGTCGAATGAAATCATACTCTTTACCT

FIGURE 4B

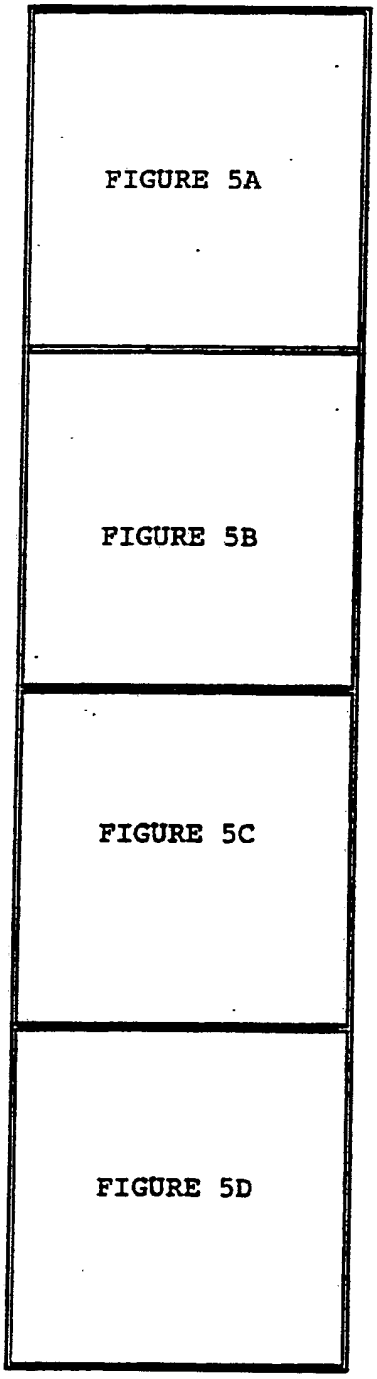
FIGURE 5A
FIGURE 5B
FIGURE 5C
FIGURE 5D

FIGURE 5A

```
                                                    CCGTTGCTGTCGAGAAAACAGAAAGAAGAGAAAA

35
ATG GAC TAC GTG AAT ATT TTG CTG GGA CTG TTT TTC ACT
Met Asp Tyr Val Asn Ile Leu Leu Gly Leu Phe Phe Thr

TGG TTC TTG GTG AAT GGA CTC ATG TCA CTT CGA AGA AGA
Trp Phe Leu Val Asn Gly Leu Met Ser Leu Arg Arg Arg

113
AAA ATC TCT AAG AAA CTT CCA CCA GGT CCA TTT CCT TTG
Lys Ile Ser Lys Lys Leu Pro Pro Gly Pro Phe Pro Leu

CCT ATC ATC GGA AAT CTT CAC TTA CTT GGT AAT CAT CCT
Pro Ile Ile Gly Asn Leu His Leu Leu Gly Asn His Pro

191
CAC AAA TCA CTT GCT CAA CTT GCA AAA ATT CAT GGT CCT
His Lys Ser Leu Ala Gln Leu Ala Lys Ile His Gly Pro

ATT ATG AAT CTC AAA TTA GGC CAA CTA AAC ACA GTG GTC
Ile Met Asn Leu Lys Leu Gly Gln Leu Asn Thr Val Val

269
ATT TCA TCA TCA GTC GTG GCA AGA GAA GTC TTG CAA AAA
Ile Ser Ser Ser Val Val Ala Arg Glu Val Leu Gln Lys

CAA GAC TTA ACA TTT TCC AAT AGG TTT GTC CCG GAC GTA
Gln Asp Leu Thr Phe Ser Asn Arg Phe Val Pro Asp Val

347
GTC CAT GTC CGA AAT CAC TCC GAT TTT TCT GTT GTT TGG
Val His Val Arg Asn His Ser Asp Phe Ser Val Val Trp

TTA CCA GTC AAT TCT CGA TGG AAA ACG CTT CGC AAA ATC
Leu Pro Val Asn Ser Arg Trp Lys Thr Leu Arg Lys Ile
```

FIGURE 5B

425
ATG AAC TCT AGC ATC TTT TCT GGT AAC AAG CTT GAT GGT
Met Asn Ser Ser Ile Phe Ser Gly Asn Lys Leu Asp Gly

AAT CAA CAT CTG AGG TCT AAA AAG GTC CAA GAG TTA ATT
Asn Gln His Leu Arg Ser Lys Lys Val Gln Glu Leu Ile

503
GAT TAT TGT CAA AAG TGT GCC AAG AAT GGC GAA GCA GTG
Asp Tyr Cys Gln Lys Cys Ala Lys Asn Gly Glu Ala Val

GAT ATA GGA AGA GCA ACT TTT GGA ACT ACT TTG AAT TTG
Asp Ile Gly Arg Ala Thr Phe Gly Thr Thr Leu Asn Leu

581
CTA TCC AAC ACC ATT TTC TCT AAA GAT TTG ACT AAT CCG
Leu Ser Asn Thr Ile Phe Ser Lys Asp Leu Thr Asn Pro

TTT TCT GAT TCT GCT AAA GAG TTT AAG GAA TTG GTT TGG
Phe Ser Asp Ser Ala Lys Glu Phe Lys Glu Leu Val Trp

659
AAC ATT ATG GTT GAG GCT GGA AAA CCC AAT TTG GTG GAC
Asn Ile Met Val Glu Ala Gly Lys Pro Asn Leu Val Asp

TAC TTT CCT TTC CTT GAG AAA ATT GAT CCG CAA GGT ATA
Tyr Phe Pro Phe Leu Glu Lys Ile Asp Pro Gln Gly Ile

737
AAG CGA CGC ATG ACT AAT AAT TTT ACT AAG TTT CTT GGC
Lys Arg Arg Met Thr Asn Asn Phe Thr Lys Phe Leu Gly

CTT ATC AGC GGT TTG ATT GAT GAC CGG TTA AAG GAA AGG
Leu Ile Ser Gly Leu Ile Asp Asp Arg Leu Lys Glu Arg

FIGURE 5C

815
AAT CTA AGG GAC AAT GCA AAT ATT GAT GTT TTA GAC GCC
Asn Leu Arg Asp Asn Ala Asn Ile Asp Val Leu Asp Ala

CTT CTC AAC ATT AGC CAA GAG AAC CCA GAA GAG ATT GAC
Leu Leu Asn Ile Ser Gln Glu Asn Pro Glu Glu Ile Asp

893 primer191
AGG AAT CAA ATC GAG CAG TTG TGT CTG GAC TTG TTT GCA
Arg Asn Gln Ile Glu Gln Leu Cys Leu Asp Leu Phe Ala

primer190
GCA GGG ACT GAT ACT ACA TCG AAT ACC TTG GAG TGG GCA
Ala Gly Thr Asp Thr Thr Ser Asn Thr Leu Glu Trp Ala

971
ATG GCA GAA CTA CTT CAG AAT CCA CAC ACA TTG CAG AAA
Met Ala Glu Leu Leu Gln Asn Pro His Thr Leu Gln Lys

GCA CAA GAA GAA CTT GCA CAA GTC ATT GGT AAA GGC AAA
Ala Gln Glu Glu Leu Ala Gln Val Ile Gly Lys Gly Lys

1049
CAA GTA GAA GAA GCA GAT GTT GGA CGA CTA CCT TAC TTG
Gln Val Glu Glu Ala Asp Val Gly Arg Leu Pro Tyr Leu

CGA TGC ATA GTG AAA GAA ACC TTA CGA ATA CAC CCA GCG
Arg Cys Ile Val Lys Glu Thr Leu Arg Ile His Pro Ala

1127
GCT CCT CTC TTA ATT CCA CGT AAA GTG GAG GAA GAC GTT
Ala Pro Leu Leu Ile Pro Arg Lys Val Glu Glu Asp Val

GAG TTG TCT ACC TAT ATT ATT CCA AAG GAT TCA CAA GTT
Glu Leu Ser Thr Tyr Ile Ile Pro Lys Asp Ser Gln Val

FIGURE 5D

1205
CTA GTG AAC GTA TGG GCA ATT GGA CGC AAC TCT GAT CTA
Leu Val Asn Val Trp Ala Ile Gly Arg Asn Ser Asp Leu

TGG GAA AAT CCT TTG GTC TTT AAG CCA GAA AGG TTT TGG
Trp Glu Asn Pro Leu Val Phe Lys Pro Glu Arg Phe Trp

1283
GAG TCA GAA ATA GAT ATC CGA GGT CGA GAT TTT GAA CTC
Glu Ser Glu Ile Asp Ile Arg Gly Arg Asp Phe Glu Leu

ATT CCA TTT GGT GCT GGT CGA AGA ATT TGC CCT GGA TTG
Ile Pro Phe Gly Ala Gly Arg Arg Ile Cys Pro Gly Leu

1361
CCT TTG GCT ATG AGG ATG ATT CCA GTA GCA CTA GGT TCA
Pro Leu Ala Met Arg Met Ile Pro Val Ala Leu Gly Ser

TTG CTA AAC TCA TTT AAT TGG AAA CTA TAT GGT GGA ATT
Leu Leu Asn Ser Phe Asn Trp Lys Leu Tyr Gly Gly Ile

1439
GCA CCT AAA GAT TTG GAC ATG CAG GAA AAG TTT GGC ATT
Ala Pro Lys Asp Leu Asp Met Gln Glu Lys Phe Gly Ile

ACC TTG GCG AAA GCC CAA CCT CTG CTA GCT ATC CCA ACT
Thr Leu Ala Lys Ala Gln Pro Leu Leu Ala Ile Pro Thr

1517
CCC CTG TAG CTATAGGGATAAATTAAGTTGAGGTTTTAAGTTACTAGT
Pro Leu

AGATTCTATTGCAGCTATAGGATTTCTTTCACCATCACGTATGCTTTACCG

TTGGATGATGGAAAGAAATATCTATAGCTTTGGGTTTGTTTAGTTTGCACA

TAAAAATTGAATGAATGGAATACCATGGAGTTATAAGAAATAATAAGACTA

TGATTCTTACCCTACTTGAACAATGACATGGCTATTTCAC

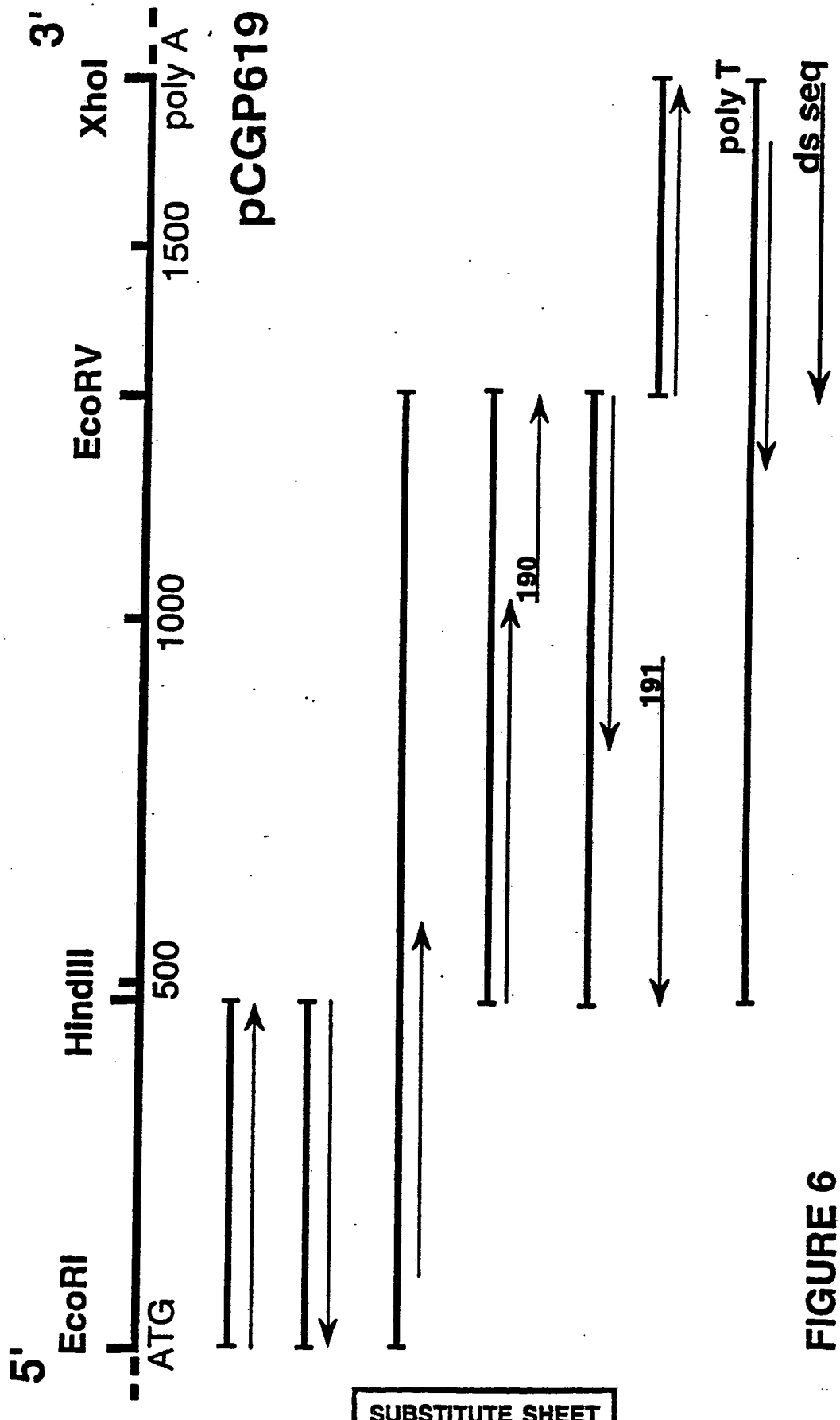
pCGP619
3'
5'
EcoRI
ATG
HindIII
EcoRV
XhoI
poly A
500
1000
1500
190
191
poly T
ds seq

FIGURE 6

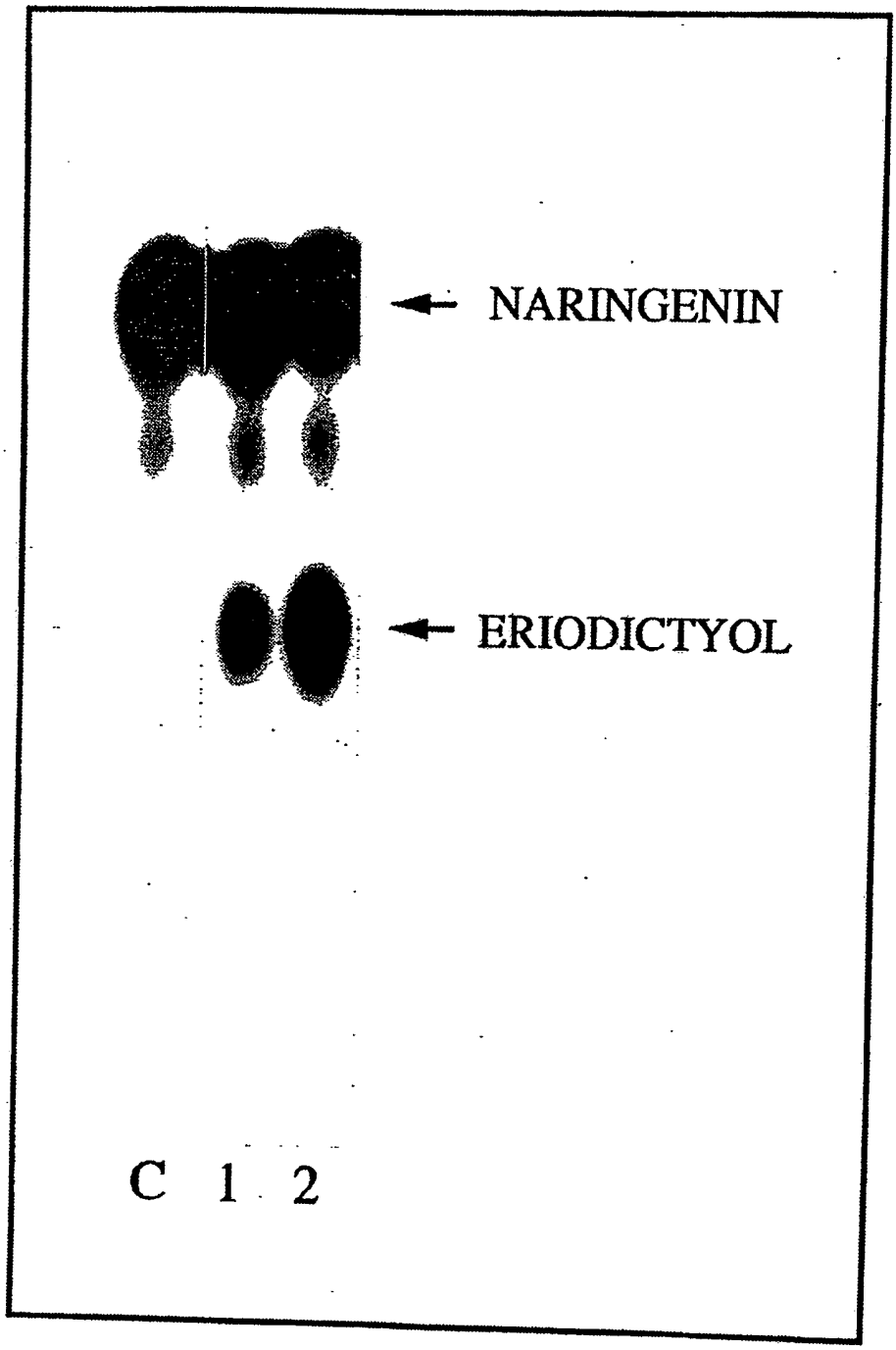

FIGURE 7

Sequence of pCGP635 insert

```
                                                                    TT
  Leu Glu Trp Ala Met Ala Glu Ile Leu Arg His Pro Arg Val Cys Arg Lys Met Gln Asn
  TTG GAG TGG GCA ATG GCC GAA ATC TTG AGG CAT CCC AGA GTT TGT AGA AAA ATG CAA AAT
3

  Glu Ala Met Glu Ile Ala Asn Gly Lys Pro His Ile Thr Glu Ser Asp Leu Asp Lys Met
  GAG GCG ATG GAG ATT GCT AAT GGC AAA CCA CAC ATC ACA GAA AGT GAT TTA GAT AAA ATG
63

  His Tyr Leu Lys Ala Val Ile Lys Glu Thr Leu Arg Leu Leu Pro Pro Ile Pro Leu Leu
  CAC TAC TTG AAA GCA GTG ATC AAA GAG ACA CTT CGG CTA CAT CCG CCA ATA CCA TTA CTC
123

  Ser Pro Arg Glu Ser Thr Glu Asp Val Lys Ile Met Glu Ser Asp Ile Glu Val Lys Lys
  TCC CCT CGT GAA TCA ACT GAA GAT GTT AAG ATA ATG GAA TCT GAC ATA GAA GTC AAA AAA
183

  Leu Trp Ser Leu Ser Met Leu Gly Gln Ser Glu Glu Thr Gln Gln Ser Gly Met Asn Gln
  CTA TGG TCT TTA TCA ATG CTT GGG CAA TCG GAA GAG ACC CAG CAG AGT GGG ATG AAC CAA
243

  Glu Phe Arg Pro Glu Arg Phe Met Asn Ser Ser Val Asp Phe Lys Gly His Leu Phe Gln
  GAG TTT CGA CCG GAG AGA TTC ATG AAT TCT TCT GTG GAT TTC AAA GGT CAT CTC TTT CAA
303

  Leu Leu Pro Phe Gly Ala Gly Arg Arg
  TTA CTC CCC TTC GGA GCC GGC CGC AGA T
363
```


FIGURE 8

SUBSTITUTE SHEET

FIGURE 9A

FIGURE 9B

Sequence of pCGP772 insert

```
   Ala Glu Leu Leu Arg Asn Pro Glu Lys Met
TG GCG GAA CTA CTG CGC AAC CCC GAG AAA ATG
            10          20          30

Ala Lys Ala Gln Asp Glu Ile Asp Arg Ile Val
GCA AAA GCA CAA GAC GAA ATA GAC CGA ATA GTA
        40          50          60

Gly Asp Lys Asn Lys Ser Phe Gln Glu Thr Asp
GGC GAC AAG AAC AAA TCG TTC CAA GAG ACA GAC
    70          80          90

Ile Ser Lys Leu Pro Tyr Ile Gln Ala Val Val
ATC TCA AAG TTA CCG TAC ATT CAA GCG GTT GTT
100         110         120         130

Lys Glu Thr Leu Arg Leu His Pro Pro Gly Pro
AAA GAA ACA TTA AGG CTA CAC CCG CCT GGA CCG
            140         150         160

Phe Leu Ile Pro His Lys Ala Glu Lys Asp Val
TTC CTA ATA CCC CAC AAA GCC GAA AAG GAC GTA
        170         180         190

Asn Leu Ser Arg Phe Phe Ile Pro Glu Asp Ala
AAC TTA AGC CGG TTT TTC ATC CCC GAG GAC GCC
    200         210         220

Gln Val Trp Val Asn Val Trp Ala Ile Gly Arg
CAA GTG TGG GTC AAT GTA TGG GCC ATT GGT CGT
230         240         250         260
```

FIGURE 9A

```
Asp Pro Ser Val Trp Arg Val Pro Leu Thr Leu
GAT CCA AGC GTG TGG CGG GTC CCA CTT ACA TTG
          270           280           290

Cys Pro Glu Arg Phe Leu Glu Asn Asp Ile Asp
TGT CCT GAA CGG TTT TTG GAA AAC GAC ATC GAT
     300           310           320

Phe Lys Gly Thr Asp Phe Glu Leu Ile Pro Phe
TTC AAA GGT ACA GAT TTC GAG CTG ATT CCC TTT
 330           340           350           360

Gly Ala Gly Arg Ile
GGC GCC GGC CGC ATC
          370
```

FIGURE 9B

FIGURE 10A

FIGURE 10B

Sequence of pCGP773 insert

```
  Met Ala Glu Leu Leu Arg Asn Pro Glu Lys Leu
A ATG GCA GAG CTG CTC CGT AAC CCA GAA AAA CTG
            10          20          30

Lys Lys Ala Gln Val Glu Leu Gln Glu Ile Ile
AAG AAA GCA CAA GTA GAG CTT CAA GAA ATC ATC
      40          50          60

Gly Arg Gly Asn Thr Leu Glu Glu Ser Asp Ile
GGC AGA GGA AAC ACA TTA GAG GAA TCT GAC ATC
 70          80          90          100

Ser Arg Leu Pro Tyr Leu Gln Ala Ile Ile Lys
AGT CGA TTG CCA TAT TTA CAG GCT ATC ATT AAG
            110         120         130

Glu Thr Phe Arg Leu His Pro Gly Leu Pro Leu
GAA ACA TTT CGG CTA CAC CCA GGA CTG CCA TTA
      140         150         160

Leu Leu Pro Arg Lys Val Gly Ser Asp Val Gln
TTG CTA CCT AGG AAA GTT GGT TCA GAC GTT CAG
    170         180         190

Leu Phe Gly Phe Thr Val Pro Lys Asn Ala Gln
CTC TTT GGG TTT ACA GTA CCC AAA AAT GCA CAA
200         210         220         230

Val Ile Ile Asn Ala Trp Ala Ile Gly Arg Asp
GTC ATA ATC AAC GCC TGG GCA ATT GGG AGA GAC
      240         250         260
```

FIGURE 10 A

SUBSTITUTE SHEET

```
Pro Asp Cys Trp Gln Lys Pro Asn Ser Phe Glu
CCA GAT TGT TGG CAG AAA CCC AAC TCA TTT GAG
     270          280          290

Pro Glu Arg Phe Leu Gly Ser Gln Ile Asp Val
CCA GAA AGG TTC CTT GGG TCA CAA ATT GAT GTG
 300          310          320          330

Lys Gly Arg Asp Phe Glu Leu Ile Pro Phe Gly
AAG GGT CGT GAT TTT GAG CTA ATT CCC TTT GGC
          340          350          360

Ala Gly Arg Ser Ile Cys Ala
GCC GGC CGC AGC ATC TGT GCC G
     370          380
```

FIGURE 10 B

Partial sequence of pCGP854 insert

PRIMER 190 --->

```
TTG GAG TGG GCA ATG GCA GAA CTT CTA CGC AAC CCG CAC ACC ATG
Leu Glu Trp Ala Met Ala Glu Leu Leu Arg Asn Pro His Thr Met
1

GCC AAA GCA AAA GAG GAG CTT AAA GAC GTT ATC GGC AAA GAA AAA
Ala Lys Ala Lys Glu Glu Leu Lys Asp Val Ile Gly Lys Glu Lys
46

CTT GTA GAT GAA GCT GAC ATT TTC GAG ACT
Leu Val Asp Glu Ala Asp Ile Phe Glu Thr
91
```

FIGURE 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU93/00127

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[5] C12N 15/53, 15/11, 9/02, C12Q 1/68, A01H 5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC C12N 15/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: C12N 15/53

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT DATABASE; WPAT; KEYWORDS FLAVAN: OR PETUNIA
DERWENT DATABASE; BIOT CHEMICAL ABSTRACTS; CASA; KEYWORDS HYDROLAS: AND (FLAVAN: OR PETUN:)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	The Journal of Biological Chemistry, volume 267, No. 8, issued March 15 1992, Bristch et al, "Molecular Cloning, Sequence Analysis, and In Vitro Expression of Flavanone 3B-Hydroxylase from Petunia hybrida" pp 5380-5387 Abstract, figure 3, page 5383, figure 5, page 5384, figure 8	1,2,4-6,14,18,22-25
Y		8-16,19,20,27-41,43-50,52-54
Y	AU,A,54123/90 (DNA PLANT TECHNOLOGY CORP) 18 October 1990 (18.10.90) Claims 12,17,22-27, page 7 line 11, page 13 line 26 to page 48 line 30	8-16,19,20,27-41,43-50,52-54

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 June 1993 (10.06.93)	29 JUNE 1993 (29.06.93)
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer R. OSBORNE Telephone No. (06) 2832313

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
P,X	AU,A,19530/92 (INTERNATIONAL FLOWER DEVELOPMENTS) 21 January 1993 page 4 lines 12-17, claims 1,2,4,5,7-11,13,15-16,18-21,23-25,27-29	1-6,8-16,22-25,31-38,47-50,47-54

This Annex lists the known [redacted] publication level patent family members [redacted]ting to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member					
AU 54123/90	WO 9011682 US 5034323	EP 465572	WO 9012084			
AU 19530/92	AU 22733/92	EP 522880	WO 9301290			
						END OF ANNEX